Identity by LC–MS

Every observed charge state of the main peak against the m/z computed from the molecular formula, under electrospray ionisation, with the criterion in parts per million and the instrument class it is meaningful for.

Not yet approved
No approval of this revision is on file. This revision states what the laboratory's results system enforces today; it takes effect when a named reviewer approves it.

Document		Laboratory	
Number	SOP-03	Name	Cupel Laboratories
Revision	1, first issue	Address	2599 Speakman Dr Mississauga, ON L5K 1B1
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Effective	Not yet in effect; takes effect when approved	Country	Canada
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Applies to	Test codes ID, IDP, FULL		
Supersedes	Nothing; first issue	Web	cupel.ca
Fingerprint	263d c9f8 d573 1bdc	Email	help@cupel.ca

1 Purpose

To confirm that the molecule present is the molecule named, by comparing the m/z of every observed charge state of the main chromatographic peak with the value computed from the molecular formula, under electrospray ionisation in positive mode.

2 Scope

The analytes of the scope table whose identity method is "LC–MS (ESI+)". Test codes ID and IDP; FULL includes it. Minimum sample, as the intake conditions state it: "one sealed vial as sold". The identity ions are acquired on the purity run's preparation (SOP-02) where both are ordered.

What a confirmation can and cannot show, as the laboratory publishes it:

> LC–MS confirms that a named molecule is present at the expected mass. It does not survey for what else may be there. It cannot distinguish stereoisomers (a D- for an L-amino acid) or sequence isomers, which have exactly the same mass. Chromatography separates some of them, and where we hold a reference standard its retention time is printed with the system suitability as the evidence for which isomer is present; where we do not, identity rests on mass alone.
>
> The limits page, "Identity is confirmation, not discovery"

3 Responsibilities

Role	Responsibility
Analyst	Acquires the run, reads the monoisotopic peak of every resolved cluster, enters the observed states and the instrument class, and signs as analyst.
Reviewer	Checks the instrument class against the criterion, the link to the HPLC main peak and the spectrum; signs the content as reviewed (SOP-07).

Named: Timothy Ian Benedicto, Medical Laboratory Technician; Roxanne Manlapaz, Laboratory Technician.

4 References and controlled sources

Standards and other documents

- IUPAC 2013 monoisotopic masses of the most abundant isotopes (the element masses below).
- SOP-02 (the chromatography and the preparation), SOP-07 (review and issue), SOP-09 (the instrument and its calibration).

Controlled sources, read at every build

- data.json scope.rows: the molecular formula of the free peptide for each analyte, from the INN monograph or PubChem, the one fact every mass is computed from; method.rows (ionisation, the identity criterion).
- chem.py: the element masses, the proton mass, the scan range.
- lab/results.py: the charge-state arithmetic, the instrument-class rule, the link to the HPLC main peak; lab/CONTRACT.md section 1 (ms).

5 Safety

- Read the current safety data sheet of every reagent before first use and keep it where the work is done. Wear a laboratory coat, safety glasses and chemical-resistant gloves for every step below.
- Treat every sample as a potent, biologically active substance of unconfirmed identity until its report says otherwise: open and weigh lyophilised powder so that it is not inhaled, and keep it off bare skin.
- Trifluoroacetic acid (in mobile phases A and B) is corrosive in its neat form and its vapour is harmful: dispense the neat acid with eye and hand protection, with ventilation that keeps the vapour from the face.
- Acetonitrile is highly flammable and toxic by inhalation, ingestion and skin contact: keep it from ignition sources and keep reservoirs capped.
- The electrospray source runs at high voltage (ESI+, 3.5 kV) and high temperature: open or clean it only with the instrument in standby, as the manufacturer’s manual requires; the source housing stays hot for minutes after standby.
- The drying and nebuliser gas is nitrogen from a cylinder (chained upright, regulator, cap on when moved, leak-tested as the supplier says) or a generator; the divert-valve line runs into the closed waste; calibrant solutions are handled as the solvents they are made in.
- Collect solvent and reagent waste in closed, labelled containers for disposal as hazardous chemical waste; never pour it to drain.
- SAF-01, the laboratory safety document (the chemical hygiene plan, the waste plan and the exposure and spill response), is published beside this procedure and governs beyond the notes here: its hazard table, the protective equipment by task and the facts it marks as not yet supplied by the owner are the controlling text; the notes here are the bench reminders of it.

6 Equipment and materials

- A liquid chromatograph coupled to a mass spectrometer with an electrospray source, under acquisition software that exports the averaged spectrum. No instrument is listed yet: make, model and serial number are published on the methods page and on each certificate once the instrument is installed and its serial plate can be photographed, and never for an instrument the laboratory does not own.
- The instrument class is recorded in the set and is required to issue, because a ppm criterion is meaningful only for a stated class. A unit-resolution class (single quadrupole, triple quadrupole, quadrupole, ion trap) cannot carry a criterion below 50 ppm; the model refuses it and says that identity on such an instrument is better stated as agreement of the average mass within 0.5–1 Da.
- Resolving power and the m/z it was measured at are recorded; a resolving power below 10 000 with a criterion below 50 ppm is a warning, since the monoisotopic peak may not be resolved above about 2 kDa.

Equipment and materials, continued

– Column, mobile phases and gradient as SOP-02.

7 Reagents and reference standards

Mobile phases as SOP-02. No reference standard is needed for the identity: the comparison is with the theoretical m/z computed from the formula. Where a reference standard is held (SOP-02), the retention time of the identity ions against the standard's main peak is the one evidence that separates isobaric forms, and VP-02 reads it.

7.1 Before the sequence: infuse or inject the calibrant (lot recorded), run the instrument's calibration or lock-mass routine, and record on WS-08 (identity run) the time, the residual error on the calibrant ions nearest the analyte's m/z and the resolving power at that m/z. Criterion: a residual within 2 ppm for a 10 ppm identity criterion (the laboratory's default). A failure is recalibrated; a second failure takes the instrument out of service (SOP-09).

8 Sample preparation

The identity solution is the purity solution 1 + 9 in mobile phase A (0.1 mg/mL), in a low-binding polypropylene vial, 2 µL injected: 0.2 µg on column, which the detector resolves without saturating, where the purity load would not. Vials consumed by the identity run are recorded as 0 when it is taken from that preparation, and the certificate says "LC–MS from the HPLC preparation". The first 1.5 min of the run are diverted to waste (the medium's salts and any Tris elute in the void). Record the dilution, the vial type and the time on WS-08 (identity run).

GHK (sold as GHK-Cu): the copper complex gives [M+Cu−H]+ ions and their charge states, which are not identity ions of the free peptide; the identity is read on the free-peptide ions only, the adducts are recorded as non-identity ions in the notes, and the divert window is shortened or the reference's alternative chromatography used where the peptide elutes in the void.

In a full panel the method as written consumes 5 vials (mass per vial 3; water content 2; HPLC from the mass per vial preparation; LC–MS from the HPLC preparation; endotoxin from the mass per vial preparation) (results.vials_required). This test takes no vial of its own there: its solution comes from the mass per vial preparation through the HPLC preparation, 0 vials are recorded as consumed by it, and the certificate says "LC–MS from the HPLC preparation". Because the endotoxin aliquot (SOP-04) is drawn from that same solution, it is made in LAL reagent water and the aliquot is taken first, before the dilution in mobile phase A that this test injects.

9 Conditions

Ionisation	ESI+, 3.5 kV
Scan range	m/z 300–2200
Identity criterion	$[M+nH]^{n+}$ within 10 ppm of theoretical
Observed peak	The monoisotopic peak of each isotope cluster (default); "most abundant" or "average" are recorded when read instead, and a ppm comparison is then flagged as not valid.
Charge states	z from 1 to 12; each observed state listed once with its m/z, intensity and acquisition retention time.
Main peak link	The ions are acquired within 0.2 min of the HPLC main peak; otherwise the certificate says which peak was identified.

10 Analytes and theoretical masses

Computed from the formula of the free peptide with the monoisotopic masses below; the analyte pages and the certificate compute the same numbers from the same formula, so the formula is the one fact to check.

C	12.00000000 Da
H	1.00782503 Da
N	14.00307400 Da
O	15.99491462 Da
S	31.97207100 Da
Cu	62.92959750 Da
Cl	34.96885268 Da
Na	22.98976928 Da
Proton	1.007276467 Da

Analyte	CAS	Formula	Monoisotopic (Da)	Note
Semaglutide	910463-68-2	$C_{187}H_{291}N_{45}O_{59}$	4111.1154	
Tirzepatide	2023788-19-2	$C_{225}H_{348}N_{48}O_{68}$	4810.5249	
Retatrutide	2381089-83-2	$C_{221}H_{342}N_{46}O_{68}$	4728.4718	CAS not yet checked against the registry; see the notes below
BPC-157	137525-51-0	$C_{62}H_{98}N_{16}O_{22}$	1418.7042	CAS not yet checked against the registry
TB-500 (Tbeta-4 17–23)	885340-08-9	$C_{38}H_{68}N_{10}O_{14}$	888.4916	CAS not yet checked against the registry; see the notes below
Ipamorelin	170851-70-4	$C_{38}H_{49}N_9O_5$	711.3857	CAS not yet checked against the registry
CJC-1295 (no DAC)	none confirmed	$C_{152}H_{252}N_{44}O_{42}$	3365.8936	see the notes below
Tesamorelin	218949-48-5	$C_{221}H_{366}N_{72}O_{67}S$	5132.7166	
Bremelanotide (PT-141)	189691-06-3	$C_{50}H_{68}N_{14}O_{10}$	1024.5243	see the notes below
Melanotan II	121062-08-6	$C_{50}H_{69}N_{15}O_9$	1023.5403	CAS not yet checked against the registry; see the notes below
AOD-9604	221231-10-3	$C_{78}H_{123}N_{23}O_{23}S_2$	1813.8604	CAS not yet checked against the registry; see the notes below
Thymosin alpha-1 (thymalfasin)	62304-98-7	$C_{129}H_{215}N_{33}O_{55}$	3106.5041	
Epitalon (AEDG)	307297-39-8	$C_{14}H_{22}N_4O_9$	390.1387	CAS not yet checked against the registry; see the notes below
GHK (sold as GHK-Cu)	49557-75-7	$C_{14}H_{24}N_6O_4$	340.1859	CAS not yet checked against the registry; see the notes below

The notes the analyte pages print, analyte by analyte:

> Retatrutide: The formula reproduces from the sequence only with one AEEA unit in the linker (gamma-Glu-AEEA-C20 diacid on Lys17); with two it would be $C_{227}H_{353}N_{47}O_{71}$.
>
> TB-500 (Tbeta-4 17–23): Two products are sold as TB-500: the acetylated fragment Ac-LKKTETQ (this row, 888.5 Da monoisotopic) and full-length thymosin beta-4 (43 residues, $C_{212}H_{350}N_{56}O_{78}S$, 4960.4863 Da monoisotopic, average 4963.44 Da, CAS 77591-33-4). Tell us which your label claims; a sample of the protein is identified against the protein's formula, not this one, and since it is not a row of this table, ask before you ship it.
>
> CJC-1295 (no DAC), the CAS number: 863288-34-0, often printed for this peptide, is an associated number of CJC-1295 with DAC ($C_{165}H_{269}N_{47}O_{46}$, 3647.19 Da), whose registry number is 446262-90-4 (CAS Common Chemistry, FDA GSRS); the no-DAC peptide, Mod GRF (1-29), has no registry number we have confirmed.
>
> CJC-1295 (no DAC): A sample of the DAC form ($C_{165}H_{269}N_{47}O_{46}$, 3645.0155 Da monoisotopic) is identified against its own formula, not this one; tell us which you have, and ask before you ship the DAC form, since it is not a row of this table.

Analytes and theoretical masses, continued

> Bremelanotide (PT-141): Melanotan II is 0.9840 Da lighter (a C-terminal amide instead of the acid), and the 13C M+1 peak of melanotan II lies 0.0193 Da (19 ppm) from this peptide's monoisotopic peak: the identity call reads the whole isotope cluster, not one peak.
>
> Melanotan II: Bremelanotide is 0.9840 Da heavier (the C-terminal acid instead of the amide), and this peptide's 13C M+1 peak lies 19 ppm from bremelanotide's monoisotopic peak: the identity call reads the whole isotope cluster, not one peak.
>
> AOD-9604: The formula is the disulfide (Cys7–Cys14) form. The reduced peptide is 2.0157 Da heavier, and its monoisotopic peak lies 4.9 ppm from the disulfide form's M+2 isotope peak: a 10 ppm match cannot exclude it, the shape of the isotope cluster can. 'HGH Fragment 176-191' as sold usually carries this same Tyr-1 sequence; native hGH 176-191 has Phe at 176 and is 15.9949 Da lighter.
>
> Epitalon (AEDG): $[M+Na]^+$ at m/z 413.128 is common for this acidic peptide and is not an identity ion. The Asp-Gly motif forms isoaspartate (same mass) and the aspartimide (−18.0106 Da).
>
> GHK (sold as GHK-Cu), the CAS number: the free tripeptide GHK; the copper complex is 89030-95-5, also not yet checked against the CAS registry.
>
> GHK (sold as GHK-Cu): GHK tripeptide; the copper complex is $C_{14}H_{22}N_6O_4Cu$. LC–MS reports the free peptide. This method confirms the GHK tripeptide and reports its purity. It does not determine whether copper is present or how much: in the acidic mobile phase the complex dissociates and the mass spectrum is that of the free peptide. A vial of GHK and a vial of GHK-Cu give the same identity result here. We do not measure copper. $[M+2H]^{2+}$ at m/z 171.1002, often the stronger GHK ion, is below the scan floor; a scan from m/z 150 would record it.

The analyte pages (data.json scope.rows)

What the mass cannot see

Each of these has an isomer of identical mass that differs only in the feature named. The identity result cannot tell them apart; where purity is also run, the retention time of the main peak against the reference standard, printed with the system suitability (SOP-02), is the only evidence the report offers, and an identity-only report has none.

Analyte	An isomer of identical mass differs only in
Tirzepatide	the lysine that carries the acyl chain: the isomer acylated on Lys16 instead of Lys20 has the same formula
Ipamorelin	the configuration of the 2-naphthylalanine and the phenylalanine at positions 3 and 4, both D (any epimer of either, and the 1-naphthyl positional isomer, has the same formula)
CJC-1295 (no DAC)	the D-alanine at position 2 (the L-Ala2 epimer is isobaric)
Bremelanotide (PT-141)	the D-phenylalanine at position 4 of the heptapeptide (7 in alpha-MSH numbering); its epimer is isobaric
Melanotan II	the D-phenylalanine at position 4 of the heptapeptide (7 in alpha-MSH numbering); its epimer is isobaric

11 Procedure

11.1 Acquire the LC–MS run on the purity preparation under the conditions above; record the instrument identifier, the data file and the acquisition time (UTC), all required to issue.

11.2 Average the spectrum from half height to half height of the main peak on the extracted-ion trace of its most abundant charge state; subtract the average of an equal window immediately before the peak; record both windows (retention times) on WS-08 (identity run) and the first as the retention time of the ions.

11.3 For each resolved isotope cluster read the monoisotopic peak: the first peak of the cluster whose spacing is 1/z, which above about 2 kDa lies several peaks left of the apex. Record z, the observed m/z, the relative intensity and the retention time. If the cluster is not resolved, record "most abundant" and the model flags the ppm comparison.

11.4 Record at least one charge state; every observed m/z must lie inside the scan range and no charge may be listed twice.

11.5 Enter the formula from the scope row; the model computes the theoretical m/z of every state and the error.

Procedure, continued

11.6 Before “Not confirmed” is entered: the calibrant error of the same day is within its criterion; the charge-state assignment agrees with the isotope spacing; the ions were taken within 0.2 min of the HPLC main peak. If the calibration fails, the run is an instrument failure, not a sample result: it is repeated after recalibration and the first file is kept and named. If all three hold, the result stands.

12 Calculations

M_{mono} = sum over elements of (count × monoisotopic mass)

$m/z_{theo}(z) = (M_{mono} + z \times 1.007276) / z$

error (ppm) = $10^6 \times (m/z_{obs} - m/z_{theo}) / m/z_{theo}$

$M_{obs}(z) = (m/z_{obs} - 1.007276) \times z$ (neutral monoisotopic mass from one state)

mean neutral mass error (Da and ppm) over the observed states

Every number on the identity page is computed from the formula and the observed m/z values; the figure draws the observed ions as sticks with the theoretical m/z of each state marked below the axis.

13 Acceptance and reporting

- Identity is Confirmed when every observed charge state lies within 10 ppm of its theoretical m/z, and Not confirmed otherwise. The criterion is the laboratory’s own specification, printed as “within 10 ppm (laboratory criterion)”; the no-specification footnote never applies to the identity row.
- The certificate prints each ion, its theoretical and observed m/z to four decimals, the error in ppm to one decimal, the neutral mass and the verdict; the formula and the monoisotopic mass to four decimals; the instrument class and resolving power; where the ions were acquired.
- An identity that does not match is reported the same way, under its number: the observed ions, the theoretical ions and the deviation. No different vial is tested and nothing is re-run until it matches; the sender is told before publication.
- The CAS number, formula and every mass refer to the free peptide. The salt form is printed as stated by the supplier or on the label, as determined, or as not determined.

14 Uncertainty

No expanded uncertainty is attached to an identity confirmation; the mass error of every charge state is reported in ppm against the criterion, with the resolving power it was measured at.

15 Deviations and open points

- A departure from this procedure, planned or found afterwards, is nonconforming work and is handled under SOP-08. One that bears on a result is stated on the certificate, in the open point or the notes of the section concerned, never left out.
- Formulas not yet checked against the monograph by a chemist: none; the analyte page says so.
- CAS numbers not yet checked against the CAS registry: Retatrutide, BPC-157, TB-500 (Tbeta-4 17–23), Ipamorelin, Melanotan II, AOD-9604, Epitalon (AEDG), GHK (sold as GHK-Cu).
- No CAS number confirmed: CJC-1295 (no DAC); the certificate prints what the scope table holds.
- The method does not survey for what else may be present; impurity masses recorded on the online run are reported under the purity open point (SOP-02).

16 Records

- The result set lab/results/<set>.json, its audit trail and its emails; the register row; the certificate and record.json as issued (SOP-07).
- WS-08 (identity by LC–MS): the calibration check, the identity solution, the averaging windows, the non-identity ions seen and the bench check before an identity is refused (SOP-07, Records and retention).
- Every observed charge state with its m/z, intensity and retention time, in the result set.
- The acquisition data file, named on the certificate with its acquisition time.

17 Revision history

Revision	Change	Approval
1	First issue.	Not yet approved

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Current revisions

End of document