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Evaluation of 2-iminoimidazolidin-4-one and thymine as respective internal standards for normal-phase and reversed-phase high-performance liquid chromatographic determination of creatinine in human serum

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Abstract

We evaluated two internal standards for HPLC determination of creatinine in human serum after ultrafiltration: 2-iminoimidazolidin-4-one for normal-phase HPLC on aluminium oxide, and thymine for C_{18} reversed-phase HPLC. Detection of 2-iminoimidazolidin-4-one was done at the same wavelength as that used for creatinine, i.e. 240 nm. For thymine, the wavelength was switched to 280 nm. The suitability of the selected compounds to serve as an internal standard in the described measurement procedures, including ultrafiltration of serum, was evaluated from the precision and accuracy obtained. The method based on normal-phase HPLC with 2-iminoimidazolidin-4-one showed an imprecision expressed as R.S.D. ranging from 0.8 to 3.4% (mean: 2.1%) and an inaccuracy, calculated from the deviations from target values determined by isotope-dilution gas chromatography–mass spectrometry, ranging from −1.3 to +1.8% (mean: +0.4%). For the reversed-phase HPLC procedure with thymine, the imprecision ranged from 0.3 to 1.3% (mean: 1.0%) and the inaccuracy from +0.1 to +3.9% (mean: +1.7%). The occasional observation of interferences with 2-iminoimidazolidin-4-one limited the application of the normal-phase method to a certain extent.

1. Introduction

Reliable determination of serum creatinine (1, Fig. 1) is still a matter of concern in clinical chemistry. Therefore great efforts have been undertaken to improve existing methods or develop new measurement principles. In particular, research has been focused on enzymatic and HPLC-based methods. In spite of the great progress made in the development of enzymatic methods (e.g. Ref. [1]), there are still applications for which simple, accurate HPLC methods are needed, like certification of reference materi-

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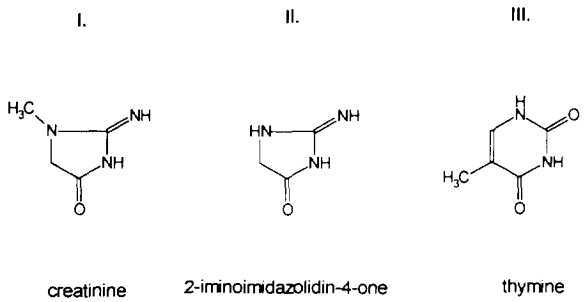

Fig. 1. Structures of creatinine (I), 2-iminoimidazolidin-4-one (II) and thymine (III). UV absorption data, λ_{max} in water at pH 7 (log ϵ) : 235 nm (3.90) (I), 223.7 nm (3.96) (II), 264.5 nm (3.90) (III).

als, routine method validation, or measurement of samples from nephrotic patients. A considerable number of HPLC methods for creatinine have already been described. Most of them use external standardization for calibration (see e.g. Refs. [2–4]), including our own approach [5]. However, a drawback of external standardization is the need for correction of the protein content after deproteinization, which is usually performed before HPLC analysis [4,5]. Furthermore, all volumetric steps have to be performed under strict control which often complicates the method [5]. Therefore, we investigated whether internal standardization could simplify the analytical protocol. From the analytical point of view, the ideal internal standard (I.S.) would have been radioactive creatinine, which indeed was successfully used in Refs. [6,7]. However, the drawbacks of using this type of I.S. are the health risk involved and the need for a liquid scintillation counter. Therefore, we did not consider this approach. Other compounds proposed for internal standardization include cimetidine [8], oxypurinol [9], allopurinol [10], phenacetine [11], and quinine dihydrochloride monohydrate [12]. Cimetidine was used in conjunction with ion-pair chromatography and thus could not be applied to the methods under investigation, i.e. aluminium oxide normal-phase (NP) and C_{18} reversed-phase (RP) HPLC. We did not consider phenacetine and quinine dihydrochloride monohydrate, because of their great chemical difference from creatinine. Since oxy- and allopurinol eluted much later than creatinine under RP-HPLC conditions comparable to the conditions used by us, we looked for compounds behaving chromatographically more similar to creatinine.

Here we report on the evaluation of 2-iminoimidazolidin-4-one (II, Fig. 1) as I.S. for aluminium oxide SP-HPLC determination of creatinine, and of thymine (III, Fig. 1) as I.S. for C_{18} RP-HPLC, both run under isocratic conditions. The accuracy of the proposed methods was investigated by analysis of 7 sera certified by isotope-dilution gas chromatography–mass spectrometry (ID-GC–MS) [13–15]. The susceptibility of these methods to interferences eluting at the position of the I.S.s was tested with patient sera.

2. Experimental

2.1. Materials and instrumentation

Guanidyl acetic acid and thymine were from Sigma (St. Louis, MO, USA). Hydrochloric acid p.a. was from Merck (Darmstadt, Germany). All solvents used for HPLC were of super purity grade (Romil Chemicals, Heidelberg, Germany). The certified lyophilized human serum based Standard Reference Materials (SRM 909 and SRM 909a) [13] and the pure creatinine SRM (SRM 914a) were obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The other lyophilized control sera were from commercial origin and certified as described in Refs. [14,15]. Reconstitution of the lyophilized sera was done on a volumetric basis [15]. Patient sera were kindly provided by the Department of Nephrology of the University Hospital of Ghent and treated according to the ethical standards in use at our University. For ultrafiltration, Amicon Centrifree filter units (Amicon Corporation, Danvers, MA, USA) were used and a Labofuge 200 (Heraeus Sepatech, Osterode, Germany). UV spectrometry was performed with a Perkin-Elmer Lambda 15 UV-Vis spectrophotometer coupled with a Lambda computer (Norwalk, CT,

USA). Mass spectra were recorded by direct inlet under electron-impact ionization (70 eV) with an HP 5988A mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). ^{13}C- and ^{1}H-NMR spectra were obtained in D_2O with external tetramethylsilane at 90 and 360 MHz, respectively, with a Bruker WH-360 instrument (Bruker, Rheinstetten, Germany) at 20°C. Weighings were done with an AT261 Deltarange balance (accuracy 0.02 mg) from Mettler Toledo (Greifensee, Switzerland). Densities were determined with a Model DMA 35 densitometer (accuracy 1 mg/ml) from A. Paar KG (Graz, Austria).

2.2. Synthesis of 2-iminoimidazolidin-4-one

2-Iminoimidazolidin-4-one was synthesized by refluxing guanidyl acetic acid in aqueous 1 *M* HCl (30 ml) for 3 h [16]. After cooling overnight and filtration, NaOH was added to precipitate unreacted substrate. The filtrate was acidified with 1 *M* HCl to pH 5.0 and water was evaporated. The residue was dissolved in methanol and filtered over silica gel (5–200 μm) to remove salts. After evaporation, the residue was crystallized from cold aqueous ammonia to afford the 2-iminoimidazolidin-4-one base.

2.3. HPLC analysis

The HPLC instrumentation used was the same as described previously [5]. It consisted of an L-6200-A Pump (Merck) equipped with a Rheodyne loop injector Model 7125 of 20 μl (Cotati, CA, USA), an L-4250 UV-Vis detector and a D-2500 Chromato Integrator (both from Merck). For SP-HPLC we used an Aluspher Al (5 μm) column (125 × 4 mm I.D., Merck), with acetonitrile–methanol (50:50, v/v) containing 7% 0.01 *M* NaOH (v/v) as eluent. RP-HPLC was performed on a Spherisorb ODS 1 (5 μm, 250 × 4.6 mm I.D.) column (PhaseSep, Norwalk, CT, USA). The eluent was 5% methanol (by vol) in 0.06 *M* $(NH_4)_2HPO_4$ (pH 7). In both cases the flow-rate was 1.0 ml/min.

2.4. Sample pretreatment and calibration

Creatinine standards were prepared in water at a concentration of 84 (A) and 464 μmol/l (B). Standard solution A was used for sera with a creatinine concentration below 220 μmol/l, solution B for sera with a concentration higher than 220 μmol/l. Both types of samples were analyzed in different analytical runs. The amount of creatinine to I.S. in the calibrators and serum samples was always adapted to give a peak-area ratio of 1, with an acceptable range of 0.9 to 1.1. Concentrations of thymine and 2-iminoimidazolidin-4-one in water were 1.19 and 6.55 mmol/l, and 1.52 and 8.36 mmol/l, respectively. A constant amount of I.S. was added to the serum samples and calibrators processed in the same run. Consequently, for serum samples, the individual volumes taken for analysis were adjusted to the creatinine concentration. For this reason, the approximate creatinine concentrations were determined prior to HPLC by a routine assay. For RP-HPLC, a serum volume of at least 100 μl was spiked with 25 μl of the thymine solution of 1.19 mmol/l for sera with a creatinine concentration below 220 μmol/l, and with 25 μl of the thymine solution of 6.55 mmol/l for sera with a concentration higher than 220 μmol/l. For NP-HPLC, serum was spiked with 25 μl of the 2-iminoimidazolidin-4-one solution of 1.52 mmol/l and with 25 μl of the 2-iminoimidazolidin-4-one solution of 8.36 mmol/l, for sera with a creatinine concentration below and higher than 220 μmol/l, respectively. All sampling steps were gravimetrically controlled. The pipetted volumes were subsequently calculated from the registered masses and densities [14]. After equilibration of the I.S. with the endogenous creatinine (12 h at 4°C or 1 h at room temperature), 100 μl of serum/I.S. mixture were ultrafiltered at 2000 *g* for 15 min. With thymine as I.S., it was necessary to wash the filter additionally with 200 μl of water. After a second centrifugation step under the same conditions, the combined ultrafiltrates were further processed. For RP-HPLC, calibrators and ultrafiltrates were directly injected and injection volumes were individually adjusted to obtain

similar detector responses. For NP-HPLC, however, it was necessary to adjust calibrators and ultrafiltrates to the eluent as described in Ref. [5], to achieve stable baseline operation and to prevent peak distortion. Adjustment was performed by an individual dilution of the samples with HPLC eluent according to their creatinine concentrations. This allowed a constant injection volume of 20 μl, giving similar detector responses for all samples.

3. Results and discussion

3.1. Evaluation of 2-iminoimidazolidin-4-one as internal standard

The identity of the synthesized 2-iminoimidazolidin-4-one was investigated by spectroscopic analysis. The UV-, ^{13}C-, ^{1}H-NMR- and mass spectrometric data were in accordance with literature data [17–19]. Used as I.S. for Aluspher Al HPLC, its retention time (t_R) was 5.07 min (t_R of creatinine: 3.10 min). The UV absorption at 240 nm of 2-iminoimidazolidin-4-one being weaker than that of creatinine, the amount of I.S. had to be adjusted to obtain comparable peak areas (the mass ratio of creatinine to I.S. is approximately 0.5). Fig. 2A shows a chromatogram of a standard mixture of creatinine and I.S. on Aluspher Al, while in Fig. 2B a representative chromatogram of a processed serum sample is shown. Chromatographically, 2-iminoimidazolidin-4-one was considered a suitable I.S.. The structural analogy between creatinine and the synthesized homologue suggested a similar behavior during ultrafiltration. This was proven by investigating the accuracy of the method. Seven serum samples were analyzed for comparison of the results with values certified by ID-GC–MS (see Table 1). The mean deviation from the certified value was +0.4% (range −1.3 to 1.8%), indicating good accuracy of the described method and hence similar behavior of creatinine and 2-iminoimidazolidin-4-one during ultrafiltration. A disadvantage of 2-iminoimidazolidin-4-one in the NP-HPLC method was its tailing peak shape, which increased the impre-

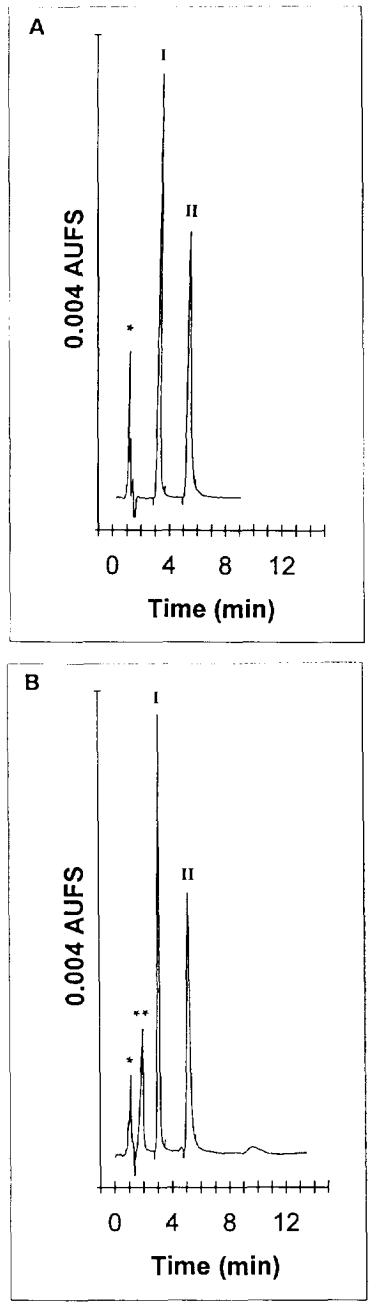

Fig. 2. Chromatogram on Aluspher Al of a standard of creatinine (84 μmol/l) (A) and of a serum sample (173 μmol/l creatinine) (B) both spiked with 2-iminoimidazolidin-4-one. The eluent consisted of 7% of 0.01 *M* (by vol) NaOH in acetonitrile–methanol (50:50, v/v). Creatinine (peak I) eluted after 3.10 min, 2-iminoimidazolidin-4-one (peak II) after 5.07 min. UV detection was done at 240 nm. The other designated peaks represent the system peaks (*) and less polar (but unidentified) endogenous serum components (**).

Table 1
Comparison of creatinine values obtained with the described HPLC methods and internal standardization with values certified by ID-GC–MS

Sample	ID-GC–MS[a] value (A) (μmol/l)	HPLC value on Aluspher with 2-iminoimidazolidin-4-one as internal standard (B) (μmol/l)	Deviation of B from A (%)	R.S.D. of B ($n = 6$) (%)	HPLC value on Spherisorb with thymine as internal standard (C) (μmol/l)	Deviation of C from A (%)	R.S.D. of C ($n = 6$) (%)
SRM 909 a1	84.0[b]	82.93	−1.3	2.8	84.49	+0.6	1.2
SRM 909 a2	436.2[b]	462.9	−0.1	0.8	479.5	+3.5	0.3
SRM 909	151.8[b]	154.6	+1.8	2.2	157.7	+3.9	1.2
1	122.8[c]	124.5	+1.4	3.4	122.9	+0.1	0.9
2	173.3[c]	173.7	+0.2	2.4	175.0	+1.0	1.1
3	165.9[c]	165.1	−0.5	1.0	167.1	+0.7	0.8
4	157.8[c]	160.2	+1.5	2.1	160.7	+1.8	1.3

[a] Isotope-dilution gas chromatography–mass spectrometry.
[b] Values certified by NIST [13].
[c] Certified as described in Refs. [14,15].

cision of the method (see also Table 1). The overall imprecision expressed as R.S.D. ($n = 6$, i.e. two serum aliquots analysed per day, and this on three consecutive days) ranged from 0.8 to 3.4% with a mean of 2.1%. In addition serum samples were analyzed without addition of I.S., which revealed, in particular for sera of nephrotic patients, the occasional presence (in ca. 20% of the samples) of interfering substances at the retention time of the I.S..

3.2. Evaluation of thymine as internal standard

The retention time of thymine on Spherisorb ODS 1 depends on the pH of the eluent. Increasing the pH resulted in a decrease of retention time. At a pH of 7, appropriate for HPLC with a silica-bonded RP stationary phase, thymine eluted at 10.65 min (t_R creatinine: 6.66 min). Thymine has two absorption maxima, at 205 and 265.5 nm. However, to avoid interferences, the wavelength for detection was set at 280 nm. Under these conditions, comparable peak areas were obtained with a mass ratio of creatinine to thymine of approximately 0.54. Fig. 3A,B shows representative chromatograms of a standard mixture and a serum sample. The detection wavelength was switched from 240 to 280 nm after 8.5 min in the same run. With regard to the ultrafiltration step of serum, to obtain accurate results it was necessary to additionally wash the filter with water and to combine the ultrafiltrates for further processing. Without this washing step, thymine was recovered to a considerably lesser degree in serum samples (appr. 10%) than creatinine. Because this was not observed for the calibrators, we assume that ultrafiltration itself is not the cause for the lower recovery of thymine, but that thymine is more strongly bound to the proteins than creatinine. The accuracy of the method can be derived from the results obtained for the certified sera as listed in Table 1. The mean deviation from the certified values was +1.7%, ranging from +0.1 to +3.9%. The mean overall imprecision expressed as R.S.D. ($n = 6$) was 1% (ranging from 0.3 to 1.3%). In contrast to the 2-iminoimidazolidin-4-one I.S., up to now no interferences at the chromatographic retention time of thymine have been observed.

4. Conclusions

The proposed internal standards generally allow reliable HPLC determination of creatinine

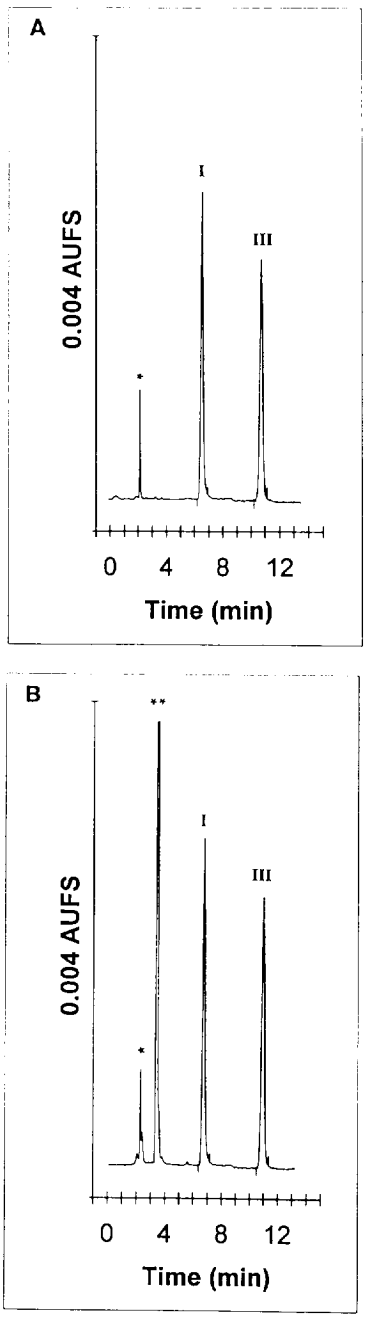

Fig. 3. Chromatogram of the same standard (A) and serum sample (B) as in Fig. 2A,B on Spherisorb ODS 1, but spiked with thymine as I.S.. The eluent consisted of 5% methanol in 0.06 *M* $(NH_4)_2HPO_4$. Creatinine (peak I) eluted after 6.66 min, thymine (peak III) after 10.65 min. The wavelength was switched from 240 to 280 nm at 8.5 min. The other designated peaks represent the system peaks (*) and more polar endogenous serum components (**).

in serum after ultrafiltration. Further, internal standardization made corrections for protein content superfluous and simplified the analytical protocol as compared to external standardization. However, the occasional occurrence of interferences when using 2-iminoimidazolidin-4-one as I.S. for NP-HPLC limited the application of this method to a certain extent. Therefore, further investigations should focus on an I.S. giving the same accuracy but allowing the choice of a more selective wavelength for UV detection.

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