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An efficient HPLC method for the analysis of isomeric purity of technetium-99m-exametazime and identity confirmation using LC–MS

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Abstract

^{99m}Tc-exametazime (^{99m}Tc-d,l-HMPAO, ^{99m}Tc-d,l-hexamethylpropyleneamine oxime) is a neutral rather unstable complex of short-lived ^{99m}Tc ($t_{1/2} = 6$ h) with the d,l-isomer (mixture of D,D- and L,L-isomers) of a bis-amine bis-oxime tetraligand. It is widely used for measurement of regional cerebral perfusion in nuclear medicine. The meso-isomer (D,L-form) should not be present in a preparation as it is not retained in brain and thus does not provide clinically useful information. Meso-HMPAO is removed from the ligand during the synthesis procedure by repeated recrystallization, but can still be present as impurity in d,l-isomer. Due to the lack of a suitable chromatographic method for analysis of the isomeric purity of ^{99m}Tc-exametazime preparations, United States Pharmacopoeia 25 (USP 25) prescribes a biological test in rats for quality control purpose. In this study, we developed a suitable high-performance liquid chromatography (HPLC) method which allows to demonstrate the relative amounts of d,l- and meso-isomer in ^{99m}Tc-exametazime and so obviates the need for a biodistribution test in animals as part of the quality control. Due to the low concentrations in which ^{99m}Tc-d,l-HMPAO is obtained (typically 2–6 ng/ml), confirmation of the identity of ^{99m}Tc-d,l-HMPAO in the monograph of the European Pharmacopoeia is now performed only indirectly by TLC and assessment of its retention time on RP-HPLC. To investigate the potential of radio-LC–MS for assessment of the identity of ^{99m}Tc-exametazime, ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO prepared using a Tc-rich eluate were analyzed using a radio-LC–MS system equipped with a time-of-flight mass spectrometer with electrospray ionization. The main peak in the radiometric channel coincided with the molecular ion mass of ^{99m}Tc-d,l-HMPAO in the mass spectrometer channel and the measured accurate mass differed only by 0.26 ppm from the theoretical mass. The identity of ^{99m}Tc-meso-HMPAO was also confirmed. Thus, radio-LC–MS allowed to obtain strong evidence for the structure of ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO at nanomolar concentration. It is concluded that radio-LC–MS can become a sensitive aid in quality control of “no carrier added” radiopharmaceutical preparations.
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Keywords: ^{99m}Tc-exametazime; ^{99m}Tc-d,l-HMPAO; ^{99m}Tc-meso-HMPAO; Diastereomers; Reversed-phase HPLC; Radio-LC–MS

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1. Introduction

Technetium-99m (^{99m}Tc)-exametazime has become a well-established and widely used radiopharmaceutical in the evaluation of regional cerebral perfusion for the diagnosis and management of various brain disorders such as stroke, vascular disease and dementia [1,2]. ^{99m}Tc is the most frequently used radionuclide for in vivo imaging studies in nuclear medicine. It has nearly ideal physical properties for use in diagnostic radiopharmaceuticals, i.e. a short half life of 6 h, gamma energy of 140 keV and no particulate emission during decay except for a limited number of conversion and Auger electrons. Combined with its useful chemical properties (technetium can be chelated by a large number of ligands) and the fact that it is continuously available from a ^{99}Mo/^{99m}Tc-generator at a reasonable cost, these characteristics make this radioisotope so popular. In ^{99m}Tc-exametazime complex, a ^{99m}Tc(V)oxo core is bound to a bis-amine bis-oxime tetraligand, commonly named hexamethylpropyleneamine oxime (HMPAO) [3]. As HMPAO ligand contains two chiral carbon atoms, it can exist in different isomeric forms, of which only the d,l-isomer should be present in ^{99m}Tc-exametazime [4]. In fact, d,l-HMPAO ligand exists as a mixture of two isomers, i.e. D,D-HMPAO and L,L-HMPAO (Fig. 1). As the meso-isomer of ^{99m}Tc-exametazime (D,L-form, Fig. 1) is not retained in brain, D,L-isomer of the ligand is removed during the

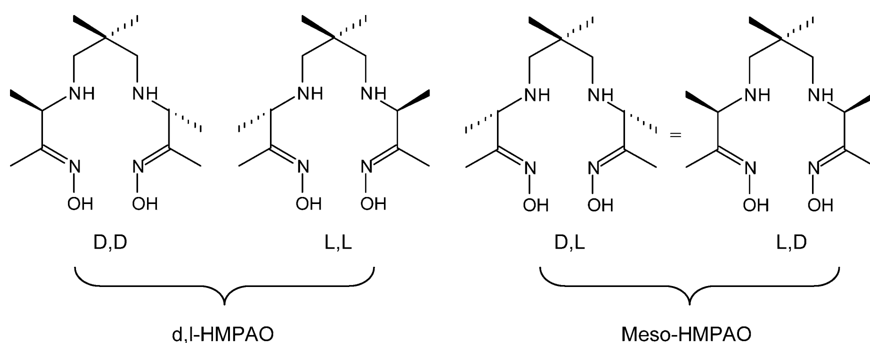

Fig. 1. Structure of HMPAO.

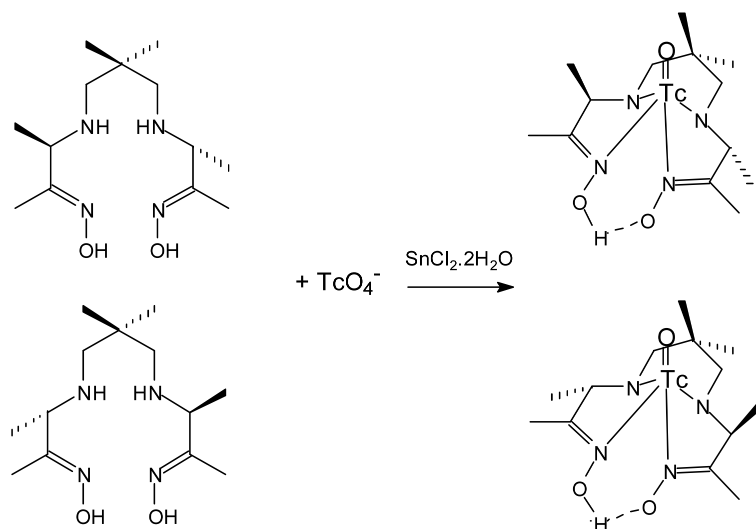

Fig. 2. Radiolabelling of d,l-HMPAO with Tc.

synthesis of HMPAO by repeated recrystallization, but it can be present as an impurity in d,l-isomer.

^{99m}Tc-exametazime can be prepared by adding eluate from a ^{99}Mo/^{99m}Tc-generator, containing ^{99m}Tc in the form of pertechnetate, to a vial containing the d,l-HMPAO ligand and a small amount of stannous ions, necessary to reduce Tc from oxidation state +7 in pertechnetate to oxidation state +5, in which form technetium is chelated by the ligand. As shown in Fig. 2, radiolabelling of d,l-HMPAO may give rise to two ^{99m}Tc-labelled enantiomers with almost identical biological properties [5]. Up to now, no chromatographic method has been reported for their separation, but in view of the similar biological behavior, there is no real need for it. On the other hand, ^{99m}Tc-meso-HMPAO—a diastereomer of ^{99m}Tc-d,l-HMPAO—does not contribute to the clinical usefulness and its relative amount in ^{99m}Tc-exametazime preparation should be limited. To our knowledge, however, no chromatographic method has yet been reported to elucidate the presence of the radiolabelled meso-isomer in ^{99m}Tc-exametazime preparation. Due to the lack of such an analytical method, the monograph on this radiopharmaceutical in USP 25 prescribes a biodistribution test in three rats as a proof of the suitability of the preparation for cerebral perfusion studies. As tests in animals should be avoided as part of a quality control procedure whenever possible, we have now studied the possibility to demonstrate the isomeric composition and purity of ^{99m}Tc-exametazime preparations using high-performance liquid chromatography (HPLC).

In generator eluate, both ^{99m}Tc and technetium-99 (^{99}Tc) are present. The mass amount of technetium (^{99m}Tc + ^{99}Tc) in preparations for clinical use is typically in the nanomole range, which is too low for analysis with conventional techniques such as UV detection or NMR. Therefore, structure elucidation of technetium complexes is currently achieved by X-ray diffraction analysis of crystals of either the identical complex synthesized on milligram scale with long-lived ^{99}Tc ($t_{1/2} = 2.12 \times 10^5$ years) or the analogous complex with stable rhenium, a chemical congener of technetium. Identity confirmation across ^{99m}Tc- and ^{99}Tc- or Re-complexes is performed by coinjection on HPLC and comparison of UV and radiometric detector signal. In the monograph for this radiopharmaceutical in the European Pharmacopoeia [6], confirmation of the identity of ^{99m}Tc-HMPAO is performed indirectly by TLC and assessment of its retention time on RP-HPLC. To our knowledge, only two reports have been published on using LC–MS for direct structure confirmation of ^{99m}Tc-labelled radiopharmaceuticals [7,8], but no report is available with regard to the analysis of ^{99m}Tc-d,l-HMPAO preparations. Therefore, the second aim of this study was to investigate whether it is possible to use radio-LC–MS as an alternative, more direct analysis tool for identification of ^{99m}Tc-d,l-HMPAO.

Additionally, we have tried to identify using this technique possible impurities in a standard ^{99m}Tc-HMPAO preparation, such as ^{99m}Tc-meso-HMPAO, which can be present when the ligand is not enantiomerically pure.

2. Materials and methods

2.1. Materials

d,l-HMPAO and meso-HMPAO were synthesized in our laboratory following a described synthesis method [9,10]. The identity of the ligands was confirmed by ^{1}H and ^{13}C NMR analyses and mass spectrometry. $SnCl_2 \cdot 2H_2O$ was commercially obtained from Merck (Darmstadt, Germany). $Na^{99m}TcO_4$ was obtained by elution of a ^{99}Mo/^{99m}Tc-generator (Ultratechnekow FM, Tyco Healthcare, Petten, The Netherlands). ^{99m}Tc-activity was measured in a Capintec CRC-35R dose calibrator (Capintec, Ramsey, NJ). The mass amount of Tc in the generator eluate is dependent on the radioactivity of ^{99m}Tc, the age of the eluate and the time interval since the previous elution of the generator and was calculated using algorithms derived for transient equilibrium generators [11].

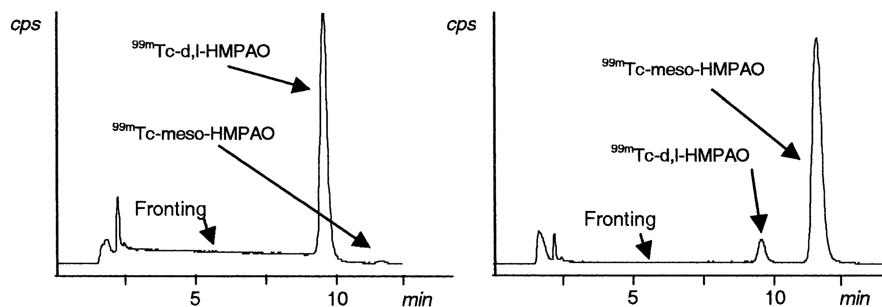

Fig. 3. HPLC chromatogram of ^{99m}Tc-d,l-HMPAO (a) and ^{99m}Tc-meso-HMPAO (b) on a Hypersil BDS column eluted isocratically with phosphate buffer (pH 3.0, 0.1 M)-acetonitrile (67:33, v/v) at a flow rate of 1.5 ml/min, 10 min after labelling.

2.2. Labelling

To a vial containing either 1 mg d,l-HMPAO or 1 mg meso-HMPAO or a mixture of 0.5 mg d,l-HMPAO and 0.5 mg meso-HMPAO in a mixture of 100 μl EtOH and 900 μl NaCl 0.9% were added consecutively 7 μg $SnCl_2 \cdot 2H_2O$ in 3.5 μl 0.05 M HCl and 400 MBq $^{99m}TcO_4^-$ in 4 ml saline. HPLC experiments were performed using eluate containing 30 pmol Tc in 400 MBq, whereas for LC–MS experiments technetium-rich eluate (130 pmol Tc in 400 MBq) was used.

2.3. High-performance liquid chromatography

HPLC equipment consisted of a Merck-Hitachi ternary gradient pump (model L-6200 intelligent pump, Merck, Overijse, Belgium) and a Valco N6 injector (Alltech, Laarne, Belgium). As stationary phases were used (a) a Hypersil ODS (5 μm) cartridge (4.6 mm × 250 mm) (Alltech), (b) a Hypersil BDS (5 μm) cartridge (4.6 mm × 250 mm) (Alltech) and (c) an XTerra™ RP18 (5 μm) cartridge (4.6 mm × 250 mm, Waters, Milford, MA). The columns were eluted isocratically at a flow rate of 1.5 ml/min with (a) mixtures of 0.1 M phosphate buffer (variable pH) and acetonitrile (67:33, v/v) or (b) mixtures of 0.1 M ammonium acetate buffer (variable pH) and acetonitrile (80:20, v/v). The column effluent was monitored for radioactivity using a 2 in. NaI(Tl) scintillation detector coupled to a single channel analyzer and a Rachel analysis program (version 1.40, Lablogic, Sheffield, UK).

2.4. Resolution

Resolution between peaks was determined with the formula of the European Pharmacopoeia [12]

$$Rs = \frac{1.18(t_{Rb} - t_{Ra})}{b_{0.5a} + b_{0.5b}}, \quad t_{Rb} > t_{Ra}$$

wherein t_{Ra} and t_{Rb} are the respective retention times, and $b_{0.5a}$ and $b_{0.5b}$ are the width of the peaks at half height.

2.5. LC–MS

The radio-LC–MS system consisted of a Waters 2690 separation module (Waters, Milford) connected to an RP C18 column (XTerra™ MS C18 3.5 μm 2.1 mm × 50 mm, Waters) eluted at a flow rate of 300 μl/min. The column eluent was monitored for absorbance using a UV detector (Waters 2487) and for radioactivity using a radiometric detector in series (3-in. NaI(Tl) detector connected to a radiation analyzer module, The Nucleus, Oak Ridge). Finally, the column eluate was directed to a time-of-flight mass spectrometer (Micromass LCT, Manchester, UK) equipped with an orthogonal ESI probe. In order to enable accurate mass calculations, the column eluate was mixed with a lock mass solution (Kryptofix® 222, Acros Organics, NJ) infused with a Harvard Instruments 22 syringe pump (Harvard Instruments, Holliston) at a flow rate of 0.1 μl/min.

The column was eluted with an isocratic mixture of acetonitrile and 0.1% formic acid in a ratio of 25:75 (v/v) at a flow rate of 300 μl/min. Aliquots of

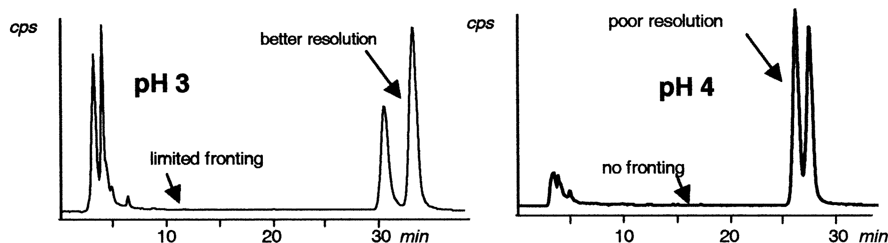

Fig. 4. HPLC chromatograms of a mixture of ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO on an XTerra column eluted isocratically with 0.1 M ammonium acetate buffer (pH 3.0 or 4.0)-acetonitrile (80:20, v/v) at a flow rate of 1.5 ml/min.

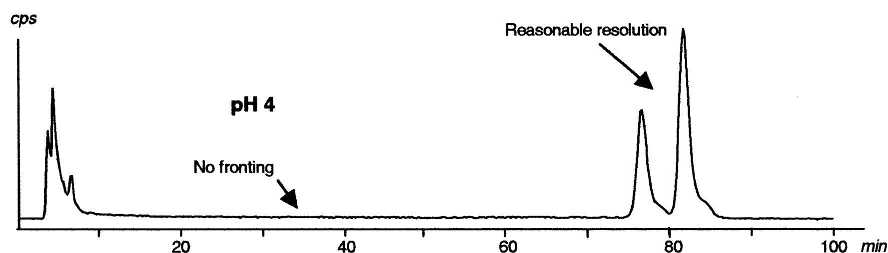

Fig. 5. HPLC chromatograms of a mixture of ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO on an XTerra column eluted isocratically with 0.1 M ammonium acetate buffer pH 4-acetonitrile (95:5, v/v) at flow rate of 1.0 ml/min.

10 μl of the labelling reaction mixture were applied on the column.

3. Results and discussion

To develop a suitable HPLC method which allows to separate the meso-isomer of ^{99m}Tc-HMPAO from d,l-isomer, different columns and eluents have been evaluated. Isocratic elution with the mixture phosphate buffer (pH 3, 0.1 M) and acetonitrile (67:33, v/v) did not result in a separation of d,l- and meso-isomer when a Hypersil ODS column was used as the stationary phase. As better results were obtained with the two other columns, the Hypersil ODS column was no longer used in further experiments. A separation of the radiolabelled d,l- and meso-isomer with a resolution >4 was obtained on the two other columns eluted with the same mobile phase as described higher for the ODS column. However, the chromatogram after labelling of d,l-isomer showed a significant fronting before the main peak, suggesting decomposition of the complex on the column (Fig. 3a). The degree of decomposition was higher on the Hypersil BDS column than on the XTerra column. This fronting was not observed in the analysis of meso-HMPAO, indicating ^{99m}Tc-meso-HMPAO is a more stable complex (Fig. 3b).

Fronting in HPLC-chromatogram and thus decomposition of ^{99m}Tc-d,l-HMPAO could be avoided by raising the pH of the mobile phase to 5 or higher, but this resulted in a clear loss of resolution.

Therefore, another mobile phase was evaluated. Using a mixture of ammonium acetate buffer (different pH values, 0.1 M) and acetonitrile (80:20, v/v) at a flow rate of 1.5 ml/min, the chromatogram of a mixture of ^{99m}Tc-HMPAO isomers (d,l+meso) showed no fronting before the peak of d,l-isomer when the pH of the mobile phase was 4 or higher (Fig. 4b). Even at pH 3, only slight fronting before the main peak was observed (Fig. 4a). Also in these conditions, decomposition was lower on the XTerra column than on the Hypersil BDS column. However, using this mobile phase the resolution between ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO was lower than with the mobile phase containing phosphate buffer. At pH 4, where no decomposition of ^{99m}Tc-d,l-HMPAO

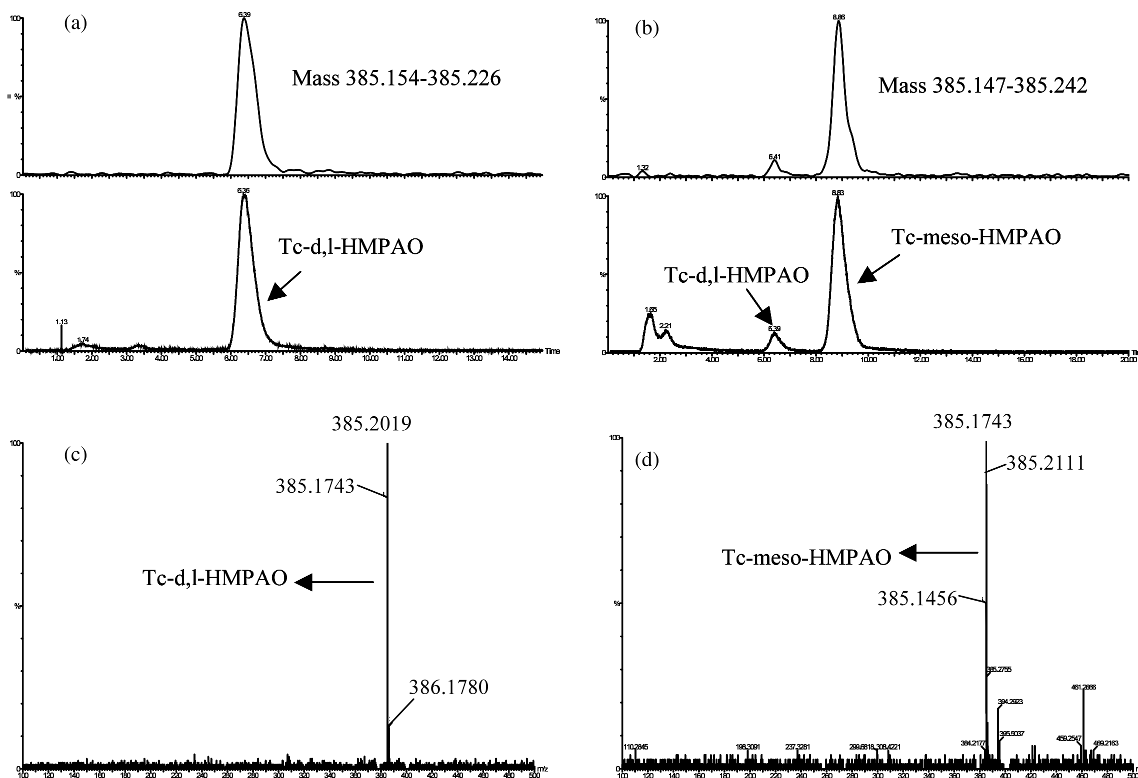

Fig. 6. Radio-LC–MS analysis of a Tc-d,l-HMPAO (a) and a Tc-meso-HMPAO preparation (c) and the background subtracted mass spectrum summed over the Tc-d,l-HMPAO peak (Rt 6.4 min) (b) and Tc-meso-HMPAO peak (Rt 8.8 min) (d).

was observed, no baseline separation could be achieved using an XTerra column eluted isocratically with a mixture of ammonium acetate buffer (pH 4, 0.1 M) and acetonitrile (80:20, v/v) at a flow rate of 1.5 ml/min.

The best compromise for separation and quantification of ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO was achieved with an XTerra column eluted isocratically with a mixture of ammonium acetate buffer (pH 4, 0.1 M) and acetonitrile (95:5, v/v) at a flow rate of 1.0 ml/min. The lowering of the relative amount of acetonitrile to 5% resulted in a serious increase of the retention time (>80 min for the meso-isomer), but on the other hand, a baseline separation was achieved with a resolution of 2.4 (Fig. 5).

For radio-LC–MS analysis purposes, the labelling kits were reconstituted with a higher concentration of technetium (130 pmol/ml) in order to facilitate mass spectrometric detection. We first used the same mobile phase as described above, but in these conditions we could not assign a molecular mass to the main peak observed on the radiometric trace of the chromatogram. Therefore, for LC–MS analysis isocratic elution was used with a mixture of acetonitrile and formic acid (0.1%, m/v) instead of ammonium acetate buffer in order to facilitate protonation of Tc-d,l-HMPAO for ES+ detection, although in these conditions some decomposition occurred. The background subtracted mass spectra summed over the main peak observed on the radiometric channel shows the presence of a peak which corresponds to the molecular ion mass of Tc-HMPAO (Fig. 6b). Accordingly, the single ion mass chromatogram corresponding to the molecular ion mass of Tc-HMPAO shows a peak with identical retention time as the main peak of the radiometric channel

(Fig. 6a). The accurate mass could be determined with a relative error of only 0.3 ppm, further providing evidence for the identity of the complex.

As meso-HMPAO is a potential impurity in the synthesis of d,l-HMPAO, we have investigated whether the technetium complex of this impurity could be identified using radio-LC–MS. Since Tc-D,L-HMPAO and Tc-meso-HMPAO are isomers, they will show identical molecular ion masses on mass spectrometric analysis and thus will need to be separated by the chromatographic system. Fig. 6c shows the analysis of the labelling reaction mixture of Tc-meso-HMPAO with a main peak on the radiometric channel eluting at 8.8 min, besides a smaller peak which elutes at the same retention time as Tc-d,l-HMPAO (6.4 min). The background corrected mass spectrum over the main peak (Rt 8.8 min) again showed a mass spectrum corresponding to the molecular ion of Tc-meso-HMPAO (Fig. 6d). The single mass chromatogram (384.15–385.24) also showed the presence of two peaks with identical retention time as the peaks of the radiometric channel (Fig. 6c). The small peak at Rt 6.4 min is probably Tc-d,l-HMPAO, generated by technetium labelling of d,l-HMPAO present as an impurity in the meso-HMPAO sample.

4. Conclusion

A suitable HPLC method was developed which allows to quantify the relative amount of the meso-isomer impurity in a ^{99m}Tc-d,l-HMPAO preparation. The newly developed HPLC method shows baseline separation between ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO with an acceptable resolution of 2.4. Moreover, no decomposition of ^{99m}Tc-d,l-HMPAO occurs during HPLC procedure. This HPLC method obviates the need for a biodistribution test in animals as part of a routine quality control procedure.

Our study also showed that the identity of ^{99m}Tc-d,l-HMPAO and ^{99m}Tc-meso-HMPAO can be confirmed using radio-LC–MS. Radio-LC–MS is thus a promising technique for confirmation of the identity of ^{99m}Tc-labelled radiopharmaceuticals both during development and for regulatory purposes.

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