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# High Performance Liquid Chromatography with Multiwavelength Detection: A Technique for Identification of Iodinated X-ray Contrast Agents in Human Body Fluids and Brain Tissue

Petter B. Jacobsen

**PURPOSE:** Several cases of a severe adverse reaction, referred to as "ascending tonic-clonic seizure syndrome," have been reported after administration of water-soluble iodinated x-ray contrast agents for myelographic examinations. Because of the bizarre reactions, the identities of the causative contrast media were questioned. **METHODS:** Analyses of biologic materials from four of these patients were performed by using reverse-phase high-performance liquid chromatography. The chromatographic system was equipped with a fast-scanning UV-visible detector for the analysis of the UV-spectra of the chromatographic peaks. **RESULTS:** The analyses revealed that the nonionic contrast agents iohexol or ioversol were not present in detectable amounts in any of the samples. On the other hand, the chromatographic analyses revealed peaks that cochromatographed with and showed the same UV-spectra as the ionic agents diatrizoate, metrizoate, and ioxitalamate. **CONCLUSION:** The results indicate inadvertent injection of ionic contrast medium in all four cases.

**Index terms:** Contrast media, effects; Liquid chromatography; Iatrogenic disease or disorder

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Several cases of a severe adverse reaction, referred to as "ascending tonic-clonic seizure (ATCS) syndrome, have been reported after administration of water-soluble iodinated contrast agents for myelographic examination (1). Whereas the nonionic low osmolar contrast agents are generally safe for intrathecal use and should have been given in all these examinations, ionic agents may give severe or fatal reactions. Hence, the identity of the given contrast agent in the reported cases (1) has been questioned, and patient samples were collected for analyses in four of the cases.

The aim of the present study was to identify possible contrast agents present in these patient samples using high-performance liquid chromatography (HPLC). Analysis of water-soluble and weakly hydrophobic nonionic contrast media in biologic materials using reversed-phase HPLC have previously been described (2-4). For the

present study, a special analytical method covering the range of possible nonionic contrast agents had to be developed, as did methods for analysis of suspected ionic agents, in samples of human brain tissue, cerebrospinal fluid (CSF), urine, plasma, and serum. As a consequence of the very difficult clinical situation, the collection of patient samples could not have been planned ahead, and the analyses had to be performed on whatever material was available after the incidents. For this reason, the preparation of the samples had to be carefully considered and tailor-made in each case.

## Materials and Methods

### *Reagents and Reference Substances*

Acetonitrile for use in the HPLC mobile phase was of HPLC grade (Merck, Darmstadt, Germany), and water was purified by reverse osmosis and Milli-Q system (Millipore, MA). All other chemicals were of analytical reagent grade.

The contrast agents used as reference samples were obtained from the following suppliers: iohexol (Omnipaque) and meglumine-,Na-,Ca-metrizoate (Isopaque) from Nycomed Imaging AS, Oslo, Norway; meglumine-,Na-diatrizoate (Hypaque) from Sterling Drug Inc, New York, NY; meglumine-,Na-ioxitalamate (Telebrix) from Byk-Gulden,

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Konstanz, Germany; meglumine-iotalamate (Conray) from Bracco Industria Chimica s.p.a., Italy; ioversol (Optiray) from Mallinckrodt Medical Inc, St. Louis, MO; iopamidol (Iopamiro) from AB Astra, Södertälje, Sweden; and meglumine-,Na-ioxaglate (Hexabrix) from Laboratoire Guerbet, Cedex, France.

Normal blood plasma from healthy volunteers was purchased from the Red Cross Blood Centre, Oslo, Norway. Nonmedicated human brain cortex tissue was kindly provided by Professor Ansgar Thorvik, Department of Neuro-pathology, Ullevål Hospital, Oslo, Norway.

### *Chromatography*

The HPLC system consisted of an SP 8800-020 ternary pump (Spectra Physics, San Jose, CA), a Gilson 232-401 Automatic sample processor and injector (Gilson Medical Electronics, Villiers-le-Bel, France) equipped with a Rheodyne 7010 injection valve (Rheodyne, Berkeley, CA) fitted with a 10  $\mu$ L fixed-volume sample loop, and a Lauda RMT6 water cooling system. The analytical reversed-phase column was a 250  $\times$  46 mm OD 5A (Spheri-5 RP-18 Brownlee Columns, Applied Biosystems, San Jose, CA) operating at ambient temperature. Two different mobile phase systems were used. The nonionic contrast agents were separated using a gradient of acetonitrile in water, whereas the ionic contrast agents were separated using isocratic acetonitrile in water acidified with H<sub>3</sub>PO<sub>4</sub>, the flow rate being 1 mL/min (see Results for details). Multiwavelength UV-detection was performed by a Spectra FOCUS Forward Optical Scanning Detector (Spectra Physics, San Jose, CA). UV-absorption was measured for the range 220–280 nm with 5-nm intervals, with a data sampling frequency of 96 points per second. Data acquisition and handling were performed by the Spectra FOCUS software on an IBM PS/2 personal computer system.

### *Preparation of Reference Samples*

Reference samples were prepared by diluting the different contrast agents with water or normal human plasma. The concentrations were adjusted to the most favorable for the respective analyses. The reference samples diluted in plasma were treated identically to the patient plasma samples.

### *Preparation of Patient Samples*

The patient samples were from Case 1, 2, 6, and 7 described in the preceding article (1). Due to the water-solubility of the contrast agents, precipitation and removal of proteins could be performed on serum and plasma (5). For samples of urine and CSF, it was sufficient to remove macromolecular components by filtration, and, from brain tissue, the contrast agent could be sampled by aqueous extraction.

**Plasma or serum** (from case 2, 6, and 7) was deproteinized by the addition of 1 volume of acetonitrile/ethanol/

water (60/38.4/1.6, v/v/v), or 1 volume of 20% (v/v) trifluoroacetic acid in water. The samples were left overnight before the proteins were removed by centrifugation at 13,000  $\times g$  for 10 minutes. All steps were carried out at 4°C. The supernatants were injected into the chromatograph either undiluted or twofold diluted in the mobile phase.

**Urine** (from case 7) was ultrafiltrated at 4°C using Centriscart I tubes (Sartorius GmbH, Göttingen, Germany) with 5000 Dalton molecular weight cut-off, or treated with acetonitrile/ethanol/water as previously described for comparison with the plasma samples from the same patient. Urine sample (200  $\mu$ L) was diluted with 800  $\mu$ L water before HPLC analysis.

**CSF** (from case 2) was analyzed untreated, except for a 50- to 200-fold dilution of the samples in water.

**Brain tissue** (from case 1, formaldehyde-fixed cortex or ependyma) was cut into small pieces and homogenized with an equal volume of saline (0.9% (w/v) NaCl in water) with a tissue homogenizer (Ikawerk, Janke & Kunkel, Staufen, Germany) for 3 minutes at 20,000 rpm at room temperature. The homogenates were centrifuged for 30 minutes at 12,000  $\times g$  at 4°C, and the supernatants were ultrafiltrated using Centriscart I tubes at 4°C before analysis.

## **Results**

Because the contrast agents possibly present in the patient samples could be either ionic or nonionic, two mobile phase systems had to be used for the HPLC analysis. The analytical approach is illustrated by describing the analysis performed on the brain tissue samples from case 1:

For the chromatographic retention of the non-ionic agents possibly present in the case 1 samples (iohexol and ioversol were used for myelographies at the hospital), a gradient of 1% to 30% (v/v) acetonitrile in water over 20 minutes was employed. Any ionic agents present (in this case possibly iotalamate or diatrizoate) would elute in the solvent front using this mobile phase. The retention of these ionic contrast agents could, however, be achieved by lowering the pH to about 2.5. This neutralization of the weak acidic groups in these ionic molecules enables a weak nonpolar/hydrophobic interaction with the stationary phase of the column, thereby making it possible to separate the ionic contrast agents using isocratic acetonitrile in water acidified with phosphoric acid.

All chromatograms are shown as multiwavelength plots, with each line representing every fifth wavelength in the range 220–280 nm (220 nm, 225 nm, 230 nm, etc). In this way, a more complete picture of the separated chromato-

graphic peaks are displayed, with more true peak heights of all the compounds. More importantly, incompletely resolved peaks may be more easily distinguished according to their peak maximal at different wavelengths.

The presence of detectable amounts of the nonionic agents iohexol or ioversol in the samples from case 1 was ruled out by comparing the chromatogram of the patient sample (Fig. 1A) with the chromatogram of the corresponding references (Fig. 1B). At the expected retention times for iohexol (11.3 minutes) and ioversol (9.9 minutes) in the chromatogram of the brain cortex extract, no significant peaks could be detected. Also analysis of the extract from brain ependyma showed similar results (data not shown).

A satisfying retention and separation of the ionic agents iotalamate and diatrizoate was obtained using an isocratic mobile phase of 3% (v/v) acetonitrile in water acidified to pH 2.5 (Fig. 2A). The chromatogram of the brain cortex extract (Fig. 2B) showed a distinct peak with retention time 8.6 minutes, identical to that of the diatrizoate reference. Similar results were obtained with the extent of brain ependyma (data not shown), whereas no peak was detected at this retention time when analyzing an extract from the cortex of a nonmedicated brain used as control (Fig. 2C).

Further comparison of the unknown compound in the brain extract with the diatrizoate reference was performed by analyzing the UV-spectra of the respective HPLC peaks. Figure 3A shows the superimposed spectra of the two peaks and also the respective first derivatives of these spectra. Full conformity of both the maximum wavelength and the shape of the spectrum is shown. This and the similar retention times strongly indicate that the two peaks represent identical substances. For comparison, the spectra

of the diatrizoate and iotalamate references are shown in Figure 3B, which demonstrates how the first derivatives can reveal and amplify small differences in the shape of the spectra.

Table 1 summarizes the results of the analyses of the samples from the four cases described in the preceding article (1). The samples from case 2, 6, and 7 were in principle analyzed the same way as described for case 1, with only minor variations concerning the mobile phases and HPLC run times. In all cases, the absence of iohexol or other nonionic agents could be demonstrated by using a mobile phase with an acetonitrile gradient and neutral pH. Also, in all cases, a positive indication on the presence of specific ionic contrast agents was obtained by performing the analysis with mobile phases at low pH.

In all four cases, the patient samples were analyzed also after addition of reference contrast agent corresponding to the unknown peak in their respective chromatograms. The resulting chromatographic peaks showed no tendencies of peak-splitting, asymmetry, or spectral changes (data not shown). Also, a factor called "peak purity," calculated by the detector system and based on the wavelength-dependent symmetry of the peaks, did not change for any of the unknown peaks after these additions. This strengthened the evidence regarding the identity of the unknown peaks.

## Discussion

The identification of unknown contrast agents by HPLC analysis was based on two independent analytical parameters, the chromatographic retention time of the compound and its UV-spectrum. The continuously recorded UV-spectra adds a "second dimension" to the analysis. In the comparison of an unknown substance with a

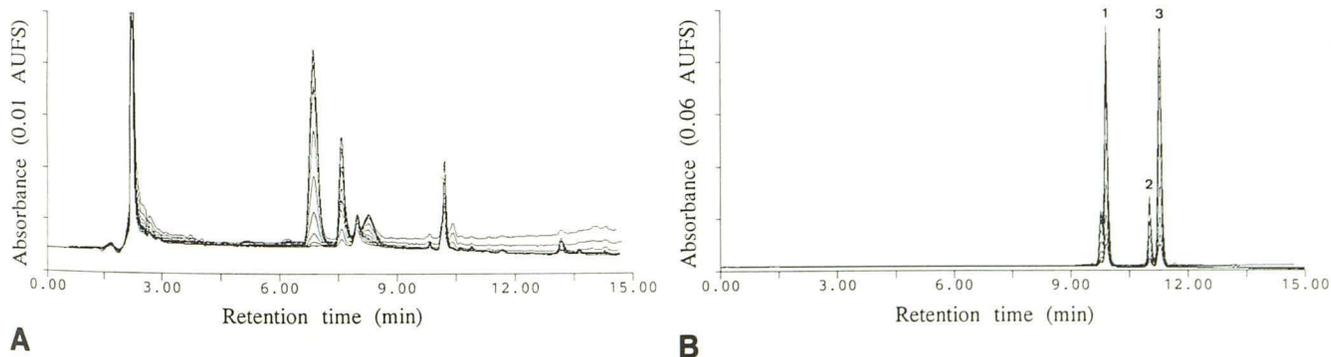


Fig. 1. Chromatograms of case 1 brain cortex extract (A) and a mixture of the iohexol and ioversol references (B). Mobile phase: 1%–30% (v/v) acetonitrile gradient, neutral pH. Detection: absorbance in the range 220–280 nm (AUFS = absorbance units full scale). Peaks in B: 1 = ioversol; 2 = iohexol endo-isomer; 3 = iohexol exo-isomer.

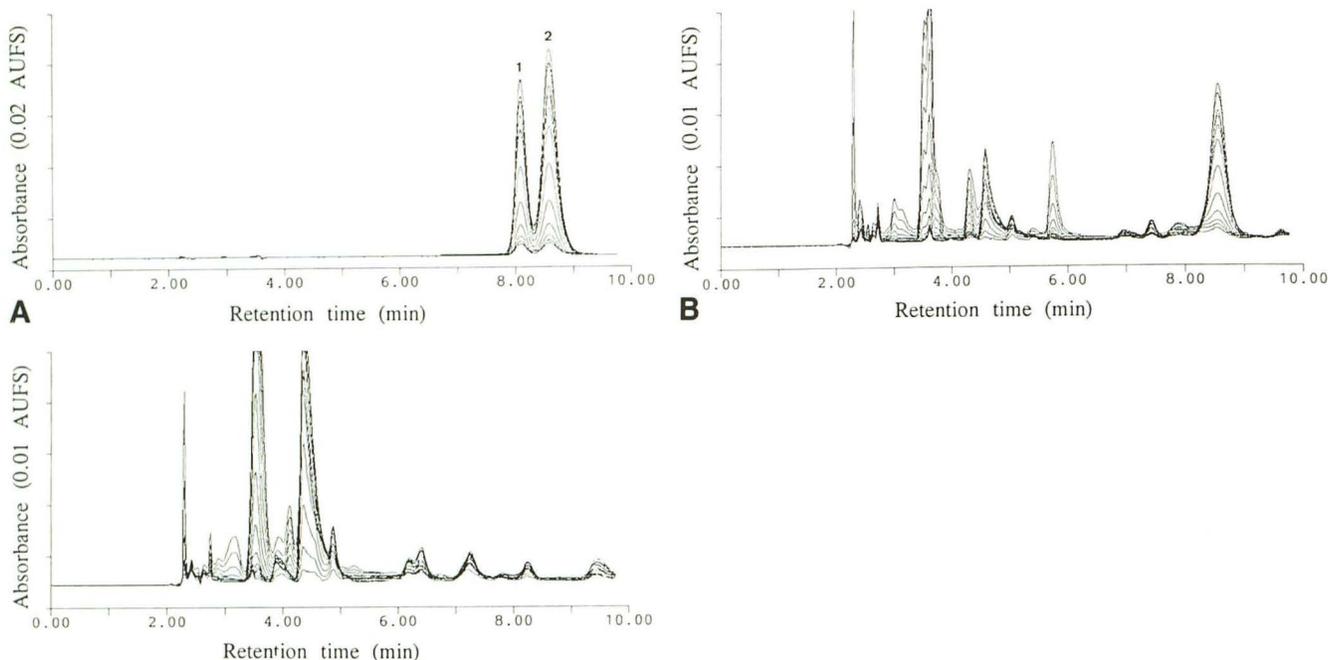


Fig. 2. Chromatograms of a mixture of iotalamate and diatrizoate references (A), case 1 brain cortex extract (B), and nonmedicated human brain cortex extract (C). Mobile phase: 3% (v/v) acetonitrile, acidified to pH 2.5 with phosphoric acid. Detection: absorbance in the range 220–280 nm (AUFS = absorbance units full scale). Peaks in A: 1 = iotalamate; 2 = diatrizoate.

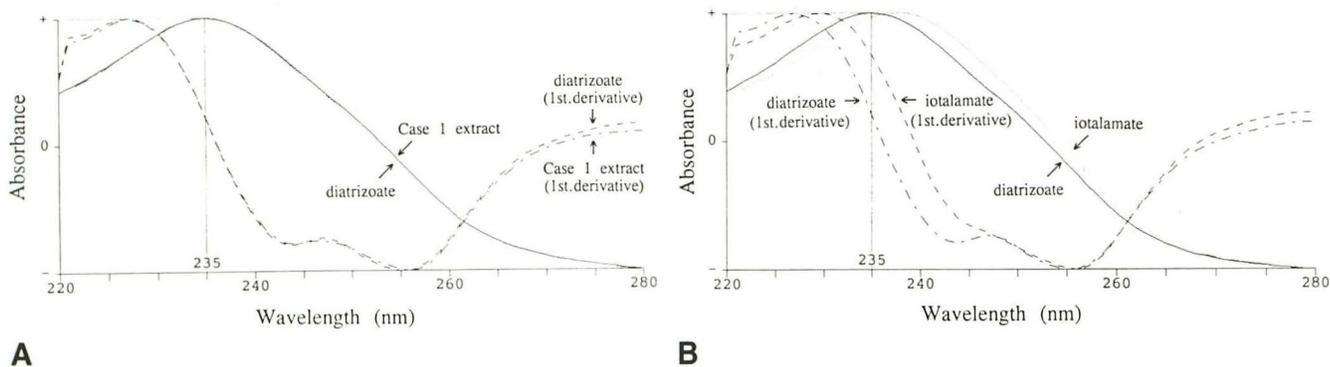


Fig. 3. UV spectrum of unknown compound in case 1 brain cortex extract compared to the reference diatrizoate spectrum (A), and the reference iotalamate spectrum compared to the reference diatrizoate spectrum (B). The *nonbroken* and *dotted lines* show the normalized spectra, whereas the *broken lines* show the normalized first derivative spectra.

known reference, full conformity for both the retention time and the spectrum is strong evidence for identification of the compound. The first derivative of the spectrum can be an especially important feature, revealing minor differences in the shape of the compared spectra, which by ordinary plotting might look very similar. This is illustrated by the fact that the slopes in the spectra of diatrizoate and iotalamate are not very different in the range 240–260 nm (Fig. 3B), but the first derivatives of these spectra have clearly different shapes in this region.

In the cases described, nonionic contrast agent approved for myelography should unquestionably have been injected, and, in all four cases, the

radiologists believed they had used iohexol. However, no traces of iohexol could be detected in any of the samples when using a mobile phase with acetonitrile gradient well suited for separation of this contrast agent from other contrast agents and endogenous compounds. On the other hand, chromatographic analyses of the samples using acidified isocratic acetonitrile mobile phases revealed the presence of peaks cochromatographing with, and showing the same UV-spectra as, the ionic contrast agents diatrizoate, metrizoate, and ioxitalamate. These results then strongly indicate inadvertent injection of ionic contrast agents in all of the four cases analyzed.

TABLE 1: Comparison of unknown compounds in the patient samples with known reference contrast agents used at the hospital<sup>a</sup>

Case No. <sup>b</sup>	Sample Type	Mobile Phase Systems	Reference Substances (Name/Retention Time (min) <sup>c</sup> )	Unknown Peak (Retention Time (min)/Reference <sup>d</sup> )
Case 1	Brain tissue <sup>e</sup>	a) 1%–30% CH <sub>3</sub> CN neutral b) 3% CH <sub>3</sub> CN pH 2.5	iohexol/11.3 <sup>f</sup> , ioversol/9.9 iotalamate/8.1, diatrizoate/8.6 <sup>f</sup>	8.6/diatrizoate
Case 2	CSF <sup>g</sup> /serum	a) 1%–30% CH <sub>3</sub> CN neutral b) 10% CH <sub>3</sub> CN pH 2.5	iohexol/11.7 <sup>f</sup> , iopamidol/9.7, ioxaglate/6.4 iopamidol/3.3, metrizoate/6.9, ioxaglate/13.0	6.8/metrizoate
Case 6	Plasma	a) 1%–15% CH <sub>3</sub> CN neutral b) 3% CH <sub>3</sub> CN pH 1.9	iohexol/12.8 ioxitalamate/6.3	6.3/ioxitalamate
Case 7	Serum/urine	a) 1%–15% CH <sub>3</sub> CN neutral b) 3% CH <sub>3</sub> CN pH 2.5	iohexol/12.8 diatrizoate in water/7.1 <sup>f</sup> , diatrizoate in serum/6.9	7.1 <sup>h</sup> /diatrizoate

<sup>a</sup> The different mobile phases used and the retention time of the references are listed. Contrast agents eluting in the solvent front (ionic agents in the gradient systems at neutral pH) are not included.

<sup>b</sup> The numbering corresponds to the cases in the preceding article (1).

<sup>c</sup> The retention time for the main isomer of the compound is given.

<sup>d</sup> Reference cochromatographing with and showing the same UV-spectrum as the unknown peak.

<sup>e</sup> Brain cortex and ependyma.

<sup>f</sup> Differences in retention times caused by analytical column of different batch numbers.

<sup>g</sup> Cerebrospinal fluid.

<sup>h</sup> In the patient urine sample.

In conclusion, HPLC can be used for reliable and fast identification of the suspected agent after unexpected adverse reactions. We propose that the analytical approach described in the present article can be used for general identification of ionic and nonionic contrast agents in human samples.

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