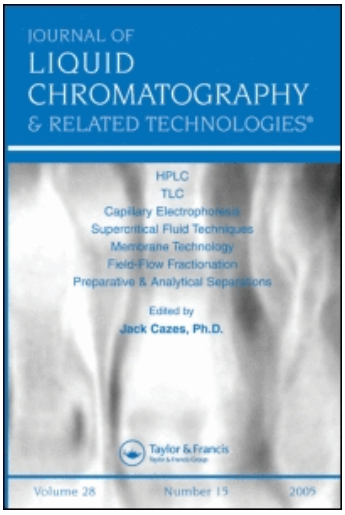

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High-Performance Liquid Chromatographic Separation and Determination of Salicylaldoxime and ß-Resorcylaldoxime in Aqueous Solutions and in Human Urine

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF SALICYLALDOXIME AND β-RESORCYLALDOXIME IN AQUEOUS SOLUTIONS AND IN HUMAN URINE

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ABSTRACT

A high-performance liquid chromatographic method for the separation and quantitation of salicylaldoxime (2-hydroxybenzaldoxime, SAO) and β-resorcylaldoxime (2,4-dihydroxybenzaldoxime, RES) is reported. Analytical methods for these compounds are needed for studies on the reactions of their antineoplastic copper(II) chelates with biomolecules and for studies on the metabolism of the chelates. A reversed-phase column (Supelcosil LC-18, 150 x 4,6 mm, 5 µm mean particle size) was used as the stationary phase and a mixture

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of tetrahydrofurane and water (volume ratio 55:45) as the mobile phase. UV absorption at 294 nm was used for detection. The retention times of RES and SAO were 4.5 min and 5.3 min, respectively, with a flow rate of 0.5 ml/min. For quantitation of SAO and RES in spiked human urine, the samples were pretreated by extracting with diethyl ether, evaporating the ether phase and dissolving the residue in the eluent. The components of the urine matrix present after this treatment did not interfere with the separation of RES and SAO. In the case of aqueous solutions as well spiked urine samples, the limit of detection of SAO was 0.2 μg/ml and that of RES 0.1 μg/ml.

INTRODUCTION

The copper(II) chelates of salicylaldoxime and its 4-hydroxy analog β-resorcylaldoxime, i.e. *trans*-bis(salicylaldoximato)copper(II) ($CuSAO_2$) and *trans*-bis(β-resorcylaldoximato)copper(II) ($CuRES_2$), as well as some analogs of them have remarkable antiproliferative activity against various tumour cell lines in cell culture [1-2] (see Fig. 1 for the structures of the compounds). $CuSAO_2$ has also been shown to have potent and in some cases even curative antitumour activity against Ehrlich ascites carcinoma in mice [1,3]. The compounds also have interesting and potentially important immunopotentiating properties *in vivo* [1,2,4] as well as *in vitro* [5]. In contrast to the chelates, the free ligands or copper(II) ions are essentially inactive against tumour cells at least *in vitro*, being devoid of antiproliferative activity in the concentration range in which $CuSAO_2$ and $CuRES_2$ totally block tumour cell proliferation [1].

Several lines of reasoning strongly suggest that $CuSAO_2$ and $CuRES_2$ are rapidly metabolized *in vivo* and possibly also *in vitro*. First, intraperitoneal administration of a chelate to rats gives rise to a visually observable green colour in the urine of the animals [4], suggesting that the copper has been liberated or that the chelate has been metabolized so that it becomes soluble in water and gets a new colour. ($CuSAO_2$ and $CuRES_2$ are almost insoluble in water and their colour is different from that observed in the urine of the treated animals.)

Second, after intraperitoneal administration of a suspension of $CuSAO_2$ or $CuRES_2$ to rats, the chelate rapidly disappears from the peritoneal cavity, in spite of its extremely low aqueous solubility. Simultaneously, the pancreata of the animals become hard and get a dark green or almost black colour becoming very easily detectable visually [4,6]. When such pancreata are put in an aqueous fixing solution, the solution is almost immediately coloured green. The colour of the exudate is different from that of either one of the chelates, suggesting again either the liberation of a metabolized form of the chelate, or the liberation of copper ions from the chelate [and possible formation of new complexes with some endogenous ligand(s)] [4,6].

Third, $CuSAO_2$ and $CuRES_2$ as well as many potent analogs of them are known to very rapidly react in the test tube with glutathione and cysteine, two common thiols present in living cells [7]. This reaction may well take place also *in vivo* and in cultured cells, and may even be involved in the mechanism of action of the chelates since the ability of glutathione and cysteine to rapidly destroy $CuSAO_2$, $CuRES_2$ and various antiproliferative and non-antiproliferative analogs is distinctly correlated with the antiproliferative activity of the compounds [7].

In order to be able to study the reactions of $CuSAO_2$ and $CuRES_2$ with thiols in more detail (e.g. to study the possible liberation of the ligands from the chelates), reliable methods are needed for the analysis of their ligand parts, salicylaldoxime (SAO) and β-resorcylaldoxime (RES) (see Fig. 1 for structural formulas of the oximes). More importantly, such methods are needed for the study of the metabolism of the chelates. Obviously, development of methods for the quantitation of the free ligands in urine is especially warranted, since the ligands or their metabolites may well be excreted in the urine. A literature study, however, does not reveal any methods for the analysis of SAO or RES. Therefore, we developed an HPLC method for the separation and quantitation of SAO and RES in aqueous solutions and in human urine.

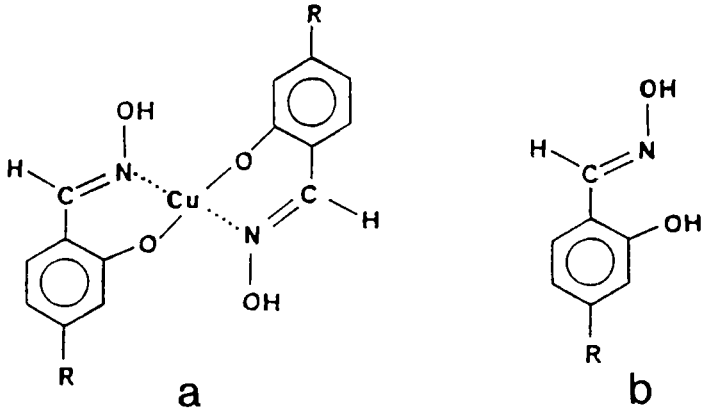

FIGURE 1 (a) The structures of $CuSAO_2$ (R = H) and $CuRES_2$ (R = OH). The systematic names of the compounds are *trans*-bis(2-hydroxybenzaldoximato)-copper(II) and *trans*-bis(2,4-dihydroxybenzaldoximato)copper(II), respectively. (b) The structures of the free ligands, SAO (R = H) and RES (R = OH). The systematic names of the ligands are 2-hydroxybenzaldoxime and 2,4-dihydroxybenzaldoxime, respectively.

EXPERIMENTAL

Chemicals

Analytical grade SAO was obtained from E.Merck (Darmstadt, Germany). RES was synthesized from 2,4-dihydroxybenzaldehyde and hydroxylamine according to standard methods. Details of the synthesis will be published elsewhere [8]. HPLC grade tetrahydrofurane (THF) was obtained from Rathburn (Walkerburn, Scotland). Water was purified by using a Millipore Alpha Q water purification system. Acetic acid was from E. Merck (Titrisol).

Urine Sample Preparation

Human urine was obtained from a 23-year-old male volunteer. Fresh samples were used daily. Urine was spiked with the aldoximes by adding an

appropriate volume of an aqueous solution (1 mg/ml) of the aldoxime to a 5-ml measuring flask and by filling with urine.

Pretreatment of Urine Samples for Chromatography

Urine samples of 1 ml volume were acidified by adding 200 µl of 0.2 M aqueous acetic acid, followed by mixing with a vortex-type shaker. The sample was then extracted once with 5 ml of diethyl ether, vortexing the tube for 1 min with the maximum effect of the mixer. The ether phase was transferred to another test tube and evaporated to dryness in a water bath (ca. 32°C) with the aid of a nitrogen stream. The residue was redissolved in 1 ml of the eluent used in the chromatography. This solution was injected into the HPLC system.

Chromatographic Apparatus and Conditions

All chromatographic measurements were carried out using a high-performance liquid chromatographic system consisting of two LC-10AD liquid chromatograph solvent delivery systems and an SPD-M6A photodiode-array UV-VIS detector (Shimadzu Corporation, Kyoto, Japan). A Hyundai Super 386N Plus computer equipped with a 120 B hard disk and 4 MB extended memory was used for data acquisition and processing in this system, employing Shimadzu LC workstation [Class-LC10 version 1]. The disk operating system was MS-DOS version 5.0. Injection was done using a Shimadzu SIL-6B auto injector, controlled by a SCL-6B system controller.

A Supelcosil LC-18 column (catalog no. 5-8230, 150 x 4.6 mm I.D., 5µm mean particle size), obtained from Supelco, Inc., Supelco Park, Bellefonte, PA, U.S.A., was employed. A LiChroCART 4-4 (Cat. 50957) precolumn containing LiChrospher 100 RP-18 packing material (particle size 5 µm) was used and was obtained from E. Merck.

Column dead volume determination was performed with the aid of 0.03 M aqueous $NaNO_3$.

Chromatographic separations were carried out at room temperature using an isocratic system with a mixture of THF and water (55:45 volume ratio) as the mobile phase. A constant flow rate of 0.5 ml/min was used. Injection volume was 20 µl. Detection was based on UV absorption at 294 nm, measured with the diode array detector. For quantitation of SAO, peak heights gave optimal results and were thus used throughout the study, while for RES, peak areas gave optimal results and were used.

RESULTS AND DISCUSSION

SAO was found to have intense absorption maxima at ca. 214, 258 and 295 nm, and RES at ca. 218 and 277 nm, and UV detection was used throughout the study. At first, attempts were done to develop methods of analysis for aqueous solutions of SAO by using methanol, ethanol or acetonitrile as such or as mixtures with water as the eluent. In these experiments, the same column was employed as in the final method developed, and the flow rate was varied between 0.5 and 1.0 ml/min. In the case of methanol-water mixtures, experiments were also performed using various column temperatures (25 - 50°C). In these studies, no satisfactory methods were found, too rapid elution and/or excessive tailing and peak broadening being the problems.

THF-water mixtures were, however, found to be suitable as the mobile phase. When THF-water mixtures were employed as the eluent, the best results were obtained with a THF/water volume ratio 55:45. With higher THF concentrations, RES and SAO eluted too rapidly considering analysis from biological matrices that may contain large amounts of various hydrophilic

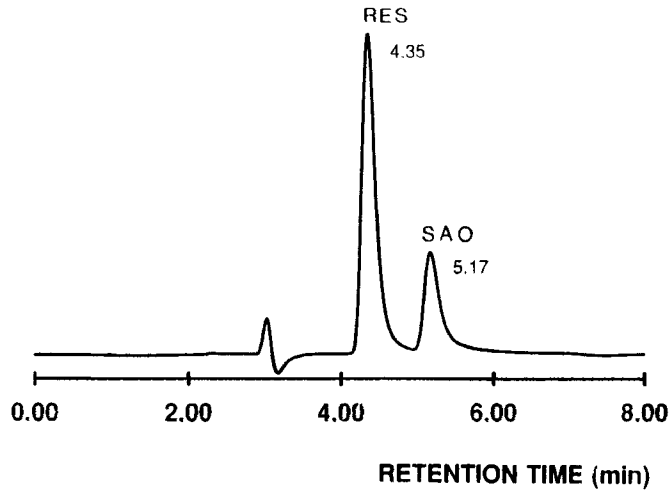

FIGURE 2. Chromatogram of an aqueous solution of SAO (12 μg/ml) and RES (8 μg/ml) (separation conditions in Experimental).

substances. With higher water contents, peak broadening caused serious problems.

With the method developed, we could also separate SAO and RES from each other (see Fig. 2), the 2,4-dihydroxylated congener RES eluting before the monohydroxylated SAO, just as would be expected. In the case of the analysis of aqueous solutions, any wavelength between ca. 200 and 300 nm could be used for detection of the analytes.

The method developed was applied also to the separation of the two congeners as well as to the quantitation of each congener in spiked human urine samples. Thus, after the pretreatment of the samples by acidification, extraction with ether, evaporation of the ether phase and redissolution of the residue, they were injected into the HPLC system. Good separation of the two aldoximes was again obtained. In the case of the pretreated urine samples, the wavelength used

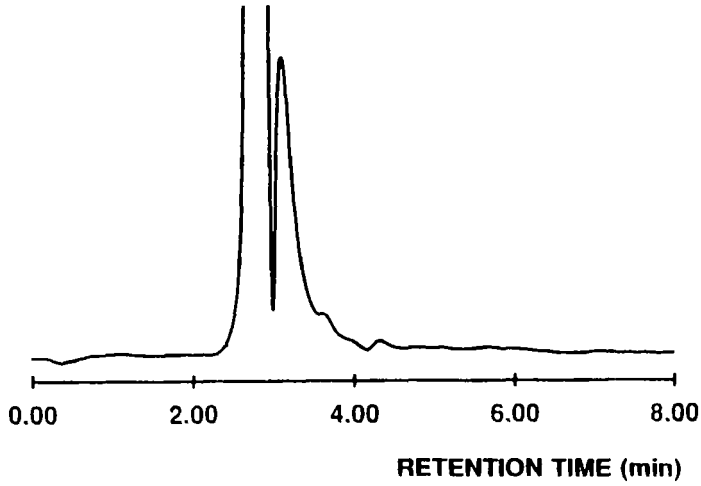

FIGURE 3. Chromatogram of a blank urine sample after the pretreatment procedure (separation conditions in Experimental).

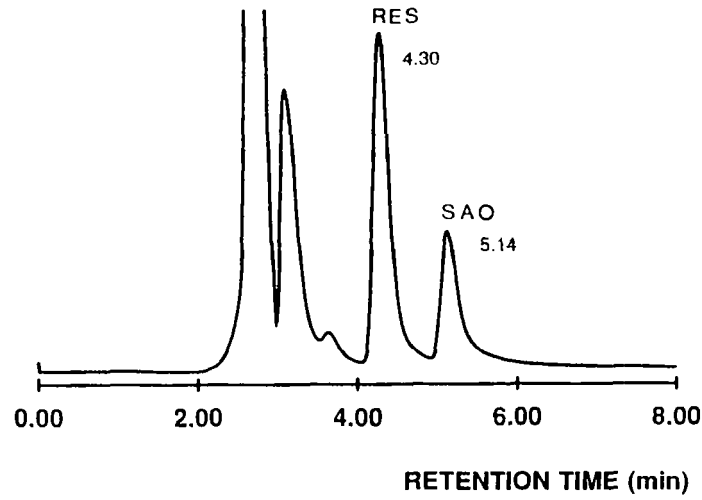

FIGURE 4. Chromatogram of urine sample spiked with SAO (20 μg/ml) and RES (10 μg/ml) and pretreated according to the method described in Experimental (separation conditions in Experimental).

TABLE 1

Recoveries of RES and SAO from spiked human urine samples.

Intra-day

Compound	Amount spiked (μg/ml)	n	Amount recovered (μg/ml) Mean	S.D.	C.V. (%)	Recovery (%)
RES	10	6	8.4	0.7	7.7	84.2
	50	6	44.8	4.5	10.1	89.6
	100	6	86.7	6.9	8.0	86.7
SAO	10	6	8.6	0.4	5.1	86.4
	50	6	42.9	2.4	5.5	85.8
	100	6	82.7	8.7	10.6	82.7

Inter-day

Compound	Amount spiked (μg/ml)	n	Amount recovered (μg/ml) Mean	S.D.	C.V. (%)	Recovery (%)
RES	10	3	8.7	0.6	7.0	87.3
	50	3	45.1	3.1	6.8	90.2
	100	3	88.2	8.8	10.0	88.2
SAO	10	3	8.7	0.7	8.5	82.0
	50	3	41.9	5.7	13.6	83.8
	100	3	84.6	8.7	10.3	84.6

for detection was found to be more critical. When the wavelength was 294 nm, absorption by the components of the pretreated urine matrix was minimal in the region of the chromatogram where RES and SAO were eluted (see Figs. 3 and 4). If detection was performed at the highest absorption maxima of SAO or RES, the matrix components of urine were not completely separated from SAO and RES, thus preventing the analysis of small concentrations of the aldoximes.

The method developed makes possible the quantitation and detection of clearly lower concentrations of RES, as compared to SAO, since the UV absorption of RES is more intense than that of SAO and also since the peak of RES is sharper than that of the slower-eluting SAO. In the case of aqueous solutions as well spiked urine, the limit of detection of SAO is 0.2 µg/ml and that of RES 0.1 µg/ml.

SAO and RES were quantitated separately using the same method as was used for the separation of the congeners. For the aqueous solutions of SAO, the method was linear in the range between 5 and 500 µg/ml ($r = 0.998$), and for those of RES between 1 and 500 µg/ml ($r = 0.999$). For the urine samples spiked with SAO, the method was linear in the range between 5 and 250 µg/ml ($r = 0.995$), and for those of RES between 1 and 100 µg/ml ($r = 0.998$). The method can, however, be used for the quantitation of higher concentrations up to ca. 1 mg/ml, if calibration is performed using several standard solutions with different concentrations. Such high concentrations, however, probably cannot exist in real urine samples.

In the case of the spiked urine samples, the mean recoveries of SAO and RES were approximately 82 - 90% (see Table 1 for details). The intra-day and inter-day repeatabilities of the method developed were acceptable (Table 1).

We found that, when dissolved in water, SAO has a strong tendency to be adsorbed on glass surfaces, necessitating the preparation of new standard solutions daily. A similar phenomenon was not observed with RES.

REFERENCES

1. H. Elo, P. Lumme, Inorg. Chim. Acta, 136: 149-153 (1987)
2. H. Elo, P. Lumme, Z. Naturforsch., 41c: 951-955 (1986)
3. H. Elo, P. Lumme, Cancer Treat. Rep., 69: 1021-1022 (1985)
4. H. Elo, I. Sunila, unpublished observations
5. H. Elo, Cancer Lett., 36: 333-339 (1987)
6. H. Elo, I. Sunila, Naturwissenschaften, 74: 245-246 (1987)
7. H. Elo, Inorg. Chim. Acta, 136: L33-L35 (1987)
8. H. Elo, manuscript in preparation

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