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ANALYTICAL STUDIES OF CONTRAST MEDIA. PART I. THIN–LAYER CHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY STUDIES

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Abstract: Conditions for thin–layer chromatographic separation of the components of Uropolinum 60% and Uropolinum 75% preparations have been determined. Products of amidotrizoic acid decomposition have been separated by using the TLC and HPLC techniques.

Keywords: amidotrizoic acid, roentgenodiagnostics, thin–layer chromatography, high performance liquid chromatography.

Amidotrizoic acid, or 3,5–bis–(acetylamino)–2,4,6–triiodobenzoic acid, is a triiodine contrast medium. A mixture of two of its salts, the sodium salt and the meglumine salt, is used in the preparations Uropolinum 60% and Uropolinum 75%, produced in Poland and utilized in X–ray examinations and CT scans of the cardiovascular system, urinary tract and organs.

The side effects associated with the administration of iodine–containing contrast media have been reported to be attributable to the following factors: high osmolarity of the preparations, the route and speed of administration, and the volume, viscosity and concentration of the contrast medium used (1–5).

Side effects may also be due to the impurities that have formed during the synthesis of amidotrizoic acid, and production or storage of the preparation.

The decomposition of amidotrizoic acid can follow various pathways: one is deacetylation of the amine groups and the resulting formation of 5–acetamido–3–amino–2,4,6–triiodobenzoic and 3,4–diamino–2,4,6–triiodobenzoic acids. Therefore the USP, BP and Ph. Eur. have recommended examining amidotrizoic acid preparations for the presence of unsubstituted amines, and the Ph. Eur. also for 5–acetamido–3–amino–2,4,6–triiodobenzoic acid.

A change of color of the substance and suspension of amidotrizoic acid following UV irradiation, i.e., distinct yellowing of the substance and browning of the suspension, suggests that iodine is splitting off the ring. The USP and BP stipulate that the substance and preparations be tested for the presence of free iodine. According to Wang, the splitting off of iodine may take place even at physiological pH (6). Wells and Juenge have suggested that the splitting off of iodine leads to the formation of two diiodine compounds, 2,4– and 2,6–diiodo substituted acids (7).

The resulting contamination can be detected by determining the amount of free iodine or the products formed after iodine split–off. The former is accomplished by determining iodine contents following incineration of a sample (8), burning it in oxygen (9, 10) or a redoxymetric procedure (11), the latter uses the chromatographic techniques HPLC and TLC or a combination of the two (7, 12–15).

The aim of this study is to optimize the conditions for chromatographic analysis of amidotrizoic acid and products of its decomposition by using TLC and HPLC techniques.

EXPERIMENTAL

Reagents and equipment

Analytical purity grade and HPLC–grade pure reagents.
Shimadzu CS 9000 densitometer;
Camag automatic TLC applicator;
Hanau quartz lamp for the decomposition procedure;
Camag quartz lamp for visualization of components on chromatograms;
Shimaduz liquid chromatograph with a UV–VIS SPD–10–A detector, LC–10AT pump, C–R6 Chrompack integrator, Rheodyne sample injector (a 7125 model with a 20 µl loop).

Material for analysis

Amidotrizoic acid, meglumine, impurities standards: 5–acetamido–3–amino–2,4,6–triiodobenzoic acid and 3,5–diamino–2,4,6–triiodobenzoic acid produced by Schering (Germany).

Thin–layer chromatography studies

In the prelliminary phase, separation of the substances making up the preparations Uropolinum 60% and 75%, i.e. amidotrizoic acid and meg-

Table 1. Results of chromatographic separation of amidotrizoic acid and meglumine by TLC technique

Mobile phase	Stationary phase	Rf values	
		amidotrizoic acid	meglumine
I	A	0.38	0.11
	B	0.80	0.24
	C	0.73	0.21
II	A	0.39	0.09
	B	0.95	0.25
	C	0.36	0.25
III	A	0.44	0
	B	0.94	0
	C	0.69	0
IV	A	0.36	0
	B	0	0
	C	0.64	0
V	A	0.33	0.02
	B	0.43	0.09
	C	0.09	0.03
VI	A	0.25	0.06
	B	0.29	0.07
	C	0.54	0.09
VII	A	0.18	0.02
	B	0.15	0.11
	C	0.45	0.05

I. 100% acetic acid : butanol : water (14 : 3 : 3)
II. chloroform : methanol : water : fromic acid (31 : 16 : 3 : 1)
III. toulene : acetone : fromic acid (5 : 5 : 1)
IV. chloroform : 1,4–dioxane – 100% acetic acid (3 : 7 : 1)
V. chloroform : methanol : 25% ammonia (10 : 5 : 1)
VI. isobutanol : isopropanol : 25% ammonia (5 : 2 : 3)
VII. butanol saturated with ammonia
A. Merck's silica gel 60 F_{254} plates
B. Merck's cellulose F plates
C. Macherey–Nagel chiral plates with fluorescent indicator

lumine, was verified by using various chromatographic systems.

Samples, 10 µl of 1% ethanolic meglumine and 2% amidotrizoic acid in a mixture of 0.1 mol/dm^{-1} sodium hydroxide–methanol (1:10 v/v) solution were placed on chromatographic plates. Chromatograms were developed by using selected mobile phases and subsequently dried at room temperature. Spots of the study substances were located by using UV irradiation and iodine vapor for amidotrizoic acid, and a 1% ninhydrine solution and iodine vapor for meglumine.

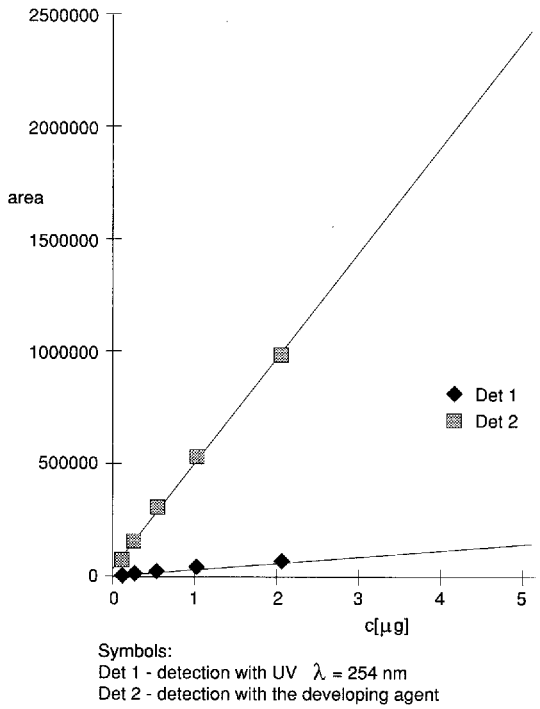


Figure 1. Diagram of linear dependence between the spot area and concentration of the substance.

Table 2. R_f values of the analyzed compounds

Compound	Mobile phase				
	II	III	V	VI	VII
amidotrizoic acid	0.44	0.42	0.31	0.31	0.20
impurity A	0.67	0.65	0.40	0.63	0.48
impurity B	0.83	0.91	2 spots 0.54 0.68	0.43	0.29
unidenitified impurities	0.52[a)]	0.48[a)]	0.48[a)]	–	0.81

A. 5-acetamido-3-amino-2,4,6-triiodobenzoic acid
B. 3,5-diamino-2,4,6-triiodobenzoic acid
[a)] visible only in UV light

Table 3. Repeatability of the selected system (n=7)

Method of localization of spots	Average response peak area, integrator units	RSD, %	Concentration µg
UVλ=254 nm	38766 ± 721	1.27	1
Developing agent	58693 ± 940	1.69	0.5

Table 4. Linear dependence between intensity and size of amidotrizoic acid spots nad the amount of the substance

Method of localization of spots	Slope a	Constant b	Linear correlation cocfficient	n	Concentration range µg
UVλ=254 nm	2.91	0.527	0.9992	5	0.2 - 5.0
Developing agent	2.79	0.684	0.9975	5	0.05 - 1.0

Table 5. Systems used for separation of amidotriozoic acid and its decomposition products

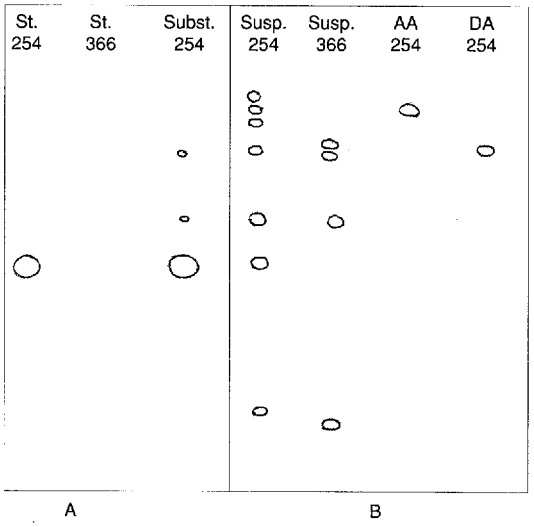
System	Stationary phase	Mobile phase	Flow ml/min	Detection nm
1	ODS Hypersil, 3 µm 60 x 4.6 mm Hewlett-Packard	water acidified with 85% phosphoric acid to pH 2.7	2	238
2	Lichrospher 100 RP-18, 5 µm 250 x 4 mm Merck	acetonitrile: water at pH 2.5 (pH adjusted with 85% phosphoric acid)	1	254
3	Lichrospher 100 RP-18, 5 µm 250 x 4 mm Merck	0.001 M solution of tetrabutylammonium diphosphate in 210:790 acetonitrile: water	1	254
4	Lichrosorb RP-18, 7 µm 125 x 4 mm Merck	0.005 M solution of tetrabutylammonium diphosphate in 210:790 acetonitrile: water	1	254

The conditions for and results of chromatographic separation of the study compounds are presented in Table 1.

On the basis of the results, the following chromatographic systems were selected for further studies: silica gel F_{254} as the stationary phase and mixtures of solvents II, III, V, VI and VII as the mobile phases.

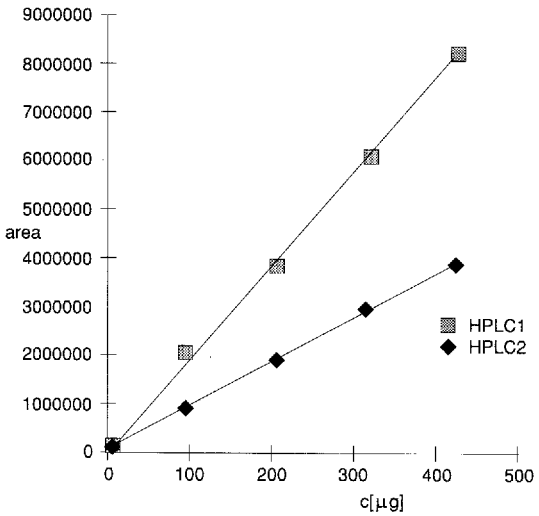
In order to obtain a material for study (decomposed amidotrizoic acid), a water suspension of amidotrizoic acid (200 mg/10 ml) was UV–irradiated (258 nm) for 21 days, and then percolated through a 0.45 µm filter.

On chromatographic plates were placed 20–µl samples of: 1) the solution containing decomposed amidotrizoic acid, 2) 0.1% solution of amidotrizoic acid in a mixture of 0.1 mol/dm^{-1} sodium hydroxide–methanol (1:10 v/v) solution, 3) 0.01% of 5–acetamido–3–amino–2,4,6–triiodobenzoic acid in a 1: 1 mixture of chloroform and methanol, and



Symbols:
St. - standard of amidotrizoic acid
Subst. - decomposed substance
Susp. - decomposed suspension
AA - 5-acetamido-3-amino-2,4,6-triiodobenzoic acid
DA - 3,5-diamino-2,4,6-triiodobenzoic acid
254, 366 - wavelengths at which chromatograms were observed

Figure 2. Chromatogram of decomposed amidotrizoic acid in the form of substance (A) and suspension (B).



Symbols:
HPLC 1 - Chromatographic system 1
HPLC 2 - Chromatographic system 2

Figure 3. Diagrams of linear dependence between the peak area and concentration of the substance.

Table 6. Results of analysis of amidotrizoic acid and products of its decomposition by high performance liquid chromatography method

System	Number of peaks detected	Retention times of detected peaks min	Retention time of amidotrizoic acid min
1	14	0.48; 0.89; 1.43; 1.68; 2.20; 2.38; 2.63; 2.98; 3.80; 5.23; 6.92; 7.46; 7.86; 10.44	5.23
2	7	1.16; 1.61; 2.04; 3.08; 3.38; 3.52; 9.47	1.61
3	12	4.38; 5.88; 6.13; 6.70; 7.30; 8.17; 8.93; 9.52; 10.68; 12.57; 13.91; 15,02	8.93
4	12	1.13; 1.35; 1.76; 2.51; 2.74; 3.28; 3.67; 4.98; 6.56; 8.39; 10.36; 15.74	3.67

Table 7. Repeatability of the selected systems (n=12)

System	Peak area	Relative standart deviation, %	Concentration, ppm
1	8286964 ± 16661	0.22	468
4	3959836 ± 18397	0.97	422

Table 8. Linear dependence between peak area and concentration of the substance (concentration range from 5 to 500 ppm)

System	Slope a	Constant b	Correlation coefficient	n
1	1.76	-0.791	0.9993	10
4	0.97	5.323	0.9996	10

4) 0.001% solution of 3,5–diamino–2,4,6–triiodobenzoic acid in a 1:1 chloroform–methanol mixture.

The chromatograms developed were dried at room temperature. Spots were localized by using UV light or a detection agent (a 2.7% solution of ferric (III) chloride in 2 mol/dm^{-1} hydrochloric acid mixed *ex tempore* with a 3.5% potassium ferricyanide (III) solution and a 3.5% sodium arsenite solution in a mixture of a 1 mol/dm^{-1} sodium hydroxide solution and 2 mol/dm^{-1} hydrochloric acid solution (6:13), at a ratio of 5:5:1).
Results are shown in Table 2.

Mobile phase II was selected as the system allowing for optimum detection and chromatographic separation of impurities in amidotrizoic acid. The spots were well–separated and compact. A detection limit for amidotrizoic acid and a determination limit (using densitometric assay) were established for the selected chromatography system. The linear dependence between the intensity and size of amidotrizoic acid spots and the amount of the substance was studied, along with the repeatability of results. These results are shown in Tables 3 and 4 and Figure 1. The relativity coefficient was below 1.7% for both detection methods.

The TLC technique was used in conditions considered to be optimal in order to compare products of amidotrizoic acid decomposition formed following UV irradiation in the substance and in its 200 mg/10 ml water suspension.
Results are shown in Figure 2.

High performance liquid chromatography studies

The conditions for HPLC analysis of amidotrizoic acid and products of its decomposition formed in a water suspension following UV irradiation were elaborated at this stage.

The following solutions were used to establish optimum conditions for chromatographic analysis: 1) a 0.04% solution of amidotrizoic acid in water, 2) the previously obtained solution of decomposed amidotrizoic acid and chromatography systems detailed in Table 5.
Study results are shown in Table 6.
Systems 1 and 4 were regarded as optimal and selected for further analyses.
Linearity and repeatability data were determined for the selected systems.
These results are shown in Tables 7 and 8, and in Figure 3.

DISCUSSION OF RESULTS

Thin–layer chromatography

Detection limit

The detection limits for amidotrizoic acid are 0.05 μg for the detection using UV irradiation and 0.01 μg for the detection using a developing agent.

Determination limit

The determination limits for amidotrizoic acid are 0.2 μg for the detection using UV irradiation and 0.05 μg for the detection using a developing agent.

Repeatability of results

The results obtained under the conditions established in the study are repeatable. Relative standard deviation is less than 1.7% for both detection methods used.

Linearity

A coefficient of correlation was obtained for each detection method. The results indicate a linear relationship between spot intensity and size and the amount of substance.

High performance liquid chromatography

Selection of mobile phase

Two mechanisms were used in the chromatographic analyses of amidotrizoic acid. The first mechanism, based on reversal of dissociation of the compound by acidification of the mobile phases with 85% phosphoric acid to a pH below 3.4 (pK_A

of amidotrizoic acid) (16) was used in systems 1 and 2. System 2 was subsequently excluded on account of too short retention times.

The other mechanism, the formation of ion pairs between amidotrizoic acid and tetrabutylammonium ions, was used in systems 3 and 4. System 4 demonstrated the better performance, and this was elected as the more adequate.

Repeatability

Both systems yielded repeatable results. The repeatability data for both systems are shown in Table 7. Relative standard deviation (RSD) is less than 1%.

Linearity

Coefficients of correlation were obtained for the selected systems. The results indicate a linear relationship between the size of the peak area and substance concentration (Table 8 and Figure 3).

CONCLUSIONS

The study has shown UV irradiation of a water suspension of amidotrizoic acid to result in the production of the greater amount of decomposition products than the action of UV radiation of the acid in the solid state, the HPLC technique being particularly useful for assessment of these processes. The conditions established for HPLC determination of the decomposition products can be used in routine analyses and in examinations of decomposed or expired preparations.

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