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Investigation on the iodination reaction of methylene blue by liquid chromatography–mass spectrometry with ionspray ionisation

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Abstract

Radioactive iodine (^{131}I and ^{123}I) labelled methylene blue is used for the early diagnosis of melanoma metastases. We studied the iodination reaction of methylene blue (using ''cold'' iodine) in order to characterise the iodination product(s) as far as number and position of iodine atoms introduced on the aromatic ring(s) is concerned. The reaction was carried out under the same experimental conditions used for the radioactive one, that is in a large excess of methylene blue. The ionspray HPLC–MS analysis of the reaction mixture showed that the iodinated methylene blue was present only in a very small amount and the main iodinated product was a demethylated one, coming out from the iodination of an impurity azure B. We also studied the iodination reaction of azure B in order to better explain the reaction pathway. Commercial azure B contains impurities of methylene blue and all the possible demethylated derivatives. HPLC–MS analysis of the reaction mixture allowed a complete characterisation of the iodinated and bis-iodinated products.

Keywords: Iodination; Pharmaceutical analysis; Methylene blue; Azure B; Dyes

1. Introduction

The use of radiolabelled pharmaceutical products is widely diffuse both in the field of metabolism investigations of new drugs and for radiodiagnosis purposes in clinical biochemistry. Radiohalogenated methylene blue has been proposed as a tracer for a sensitive and specific scintigraphic method to detect metastastic disease in melanoma patients, owing to its affinity for natural and synthetic melanin [1,2].

Methylene blue [3,7-bis(dimethylamino)-phenazathionium chloride, **1**, Fig. 1] is a coplanar polycyclic aromatic basic dye that belongs to the thiazin class. The interactive forces between **1**and the melanin polymer appear to be due to a charge transfer reaction, rather than covalent or ionic bonds [3]. Compound **1** labelled with ^{211}Astatine has been shown to be effective for therapeutic radiotherapy for pigmented melanoma in animal model systems [4], and radiolabelled **1** with iodine-123 has been used for comparative biodistribution studies in man and animals for targeting of disseminated melanoma [5]. Since the main characteristic of pigmented cells is melanogenesis, which occurs in both normal and neoplastic cells [6], the selectivity of the diagnostic method is due to the high concentration and prolonged retention of **1** in melanoma cells, while in

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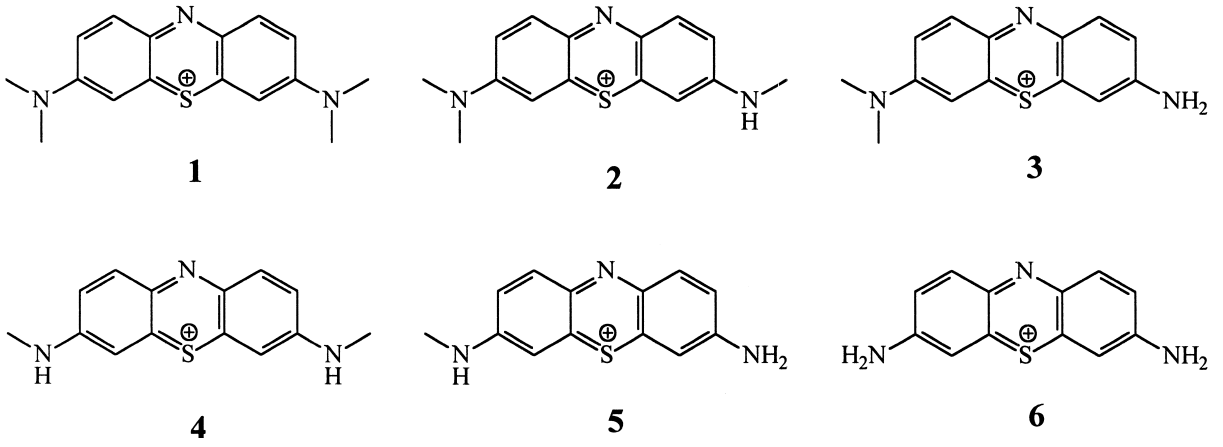

Fig. 1. Structure of methylene blue (**1**) and of the other derivatives present in the samples examined (**2**–**6**).

other tissues and organs a rapid clearance of radiolabelled **1** takes place [7,8]. However, current labelling methods for methylene blue give a final yield no better than 60–70% following 1 h of reaction time [9,10]. In the Nuclear Medicine Division of the University of Pisa they developed a fast and practical labelling method to synthesize covalently radiohalogenated **1**, both with and without cold carrier added, taking into consideration the instability of **1** with alkali iodides [11]. This rapid and efficient method of labelling allows to obtain a ready for use tracer solution with a final yield of 85–90% (with respect to the incorporation of radiolabelled iodine) and high reproducibility in the shorter time of 45 min.

This radiolabelling method of **1** uses electrophilic aromatic substitution. The presence of electron donating amino groups (3 and 7 position) should induce an *ortho* directing substitution. The main target of this research was the characterisation of the iodination product(s) by the study of the cold-analogous reaction with ^{127}I by means of high performance liquid chromatography coupled with mass spectrometry (HPLC–MS) with ionspray ionisation (ISI). Preliminary experiments showed that the main iodinated product present in the reaction mixture was a demethylated species, corresponding both to a demethylation occurring during the iodination reaction or to the iodination of the impurity, azure B (**2**). We hence decided to investigate also the iodination reaction of azure B and check for all the possible species with different degrees of methylation (**1**–**6**), both in the starting materials and in the reaction mixtures, as well as their mono or poly-iodinated derivatives.

2. Experimental

2.1. Materials and methods

Methylene blue, sterile, and pyrogen free aqueous solution 100 mg/10 ml (S.A.L.F., Bergamo, Italy) for i.v. was used as supplied (composition in 10 ml: methylene blue 100 mg, glucose 500 mg), as was azure B (Sigma–Aldrich, Milwaukee, WI, USA). All the solutions were prepared using sterile pyrogen free water for injection FU (Bieffe Medical, Sondrio, Italy). The reaction solutions were prepared using potassium iodide (''Baker Analysed'', J.T. Baker, Deventer, The Netherlands), potassium iodate (Merck, Darmstadt, Germany), and hydrochloric acid 37% (RPE grade, Carlo Erba, Milan, Italy). The mobile phase for HPLC was prepared with formic acid (purum) 98% (Fluka, Buchs, Switzerland), ammonium formate (Fluka), and acetonitrile chromasolv (Riedel-de Haen, Seelze, Germany). The solution was adjusted to physiological pH (7.4) by opportune additions of 1 *M* sodium hydroxide (Merck), potassium dihydrogenphosphate (Merck) and disodium hydrogenphosphate (J.T. Baker).

All the reactions were carried out exactly under the same conditions and using the same procedures as when radioactive iodine was used. In particular,

all the solutions were freshly prepared just prior the reaction, and the final reaction mixtures were immediately analysed.

2.2. Instrumentation

The reaction was carried out at 100°C using a thermostat controlled aluminium heating block (FALC Instruments, Bergamo, Italy).

The HPLC system consisted of a Perkin-Elmer Series 200 four solvents programmable delivery pump (Perkin-Elmer, Norwalk, CT, USA) equipped with a Perkin-Elmer Series 200 autosampler, and a Waters Bondapak C_{18} 300×3.9 mm column, 5 μm particle size (Waters, Milford, MA, USA). For preliminary investigations (see chromatograms in Fig. 2 and 4), a Perkin-Elmer Pecosphere C_{18} 30× 4.2 mm, 3 μm particle size, and a gradient elution of 30 min total time were used. The separation was carried out under gradient conditions: phase A: ammonium formate–formic acid 0.625×10^{-2} *M*; phase B: acetonitrile. The linear gradient was defined as follows: $t=0$ $A=100\%$; $t=5$ min $A=100\%$; $t=25$ min $A=30\%$ up to the end ($t=35$ min). The flow rate was 1 ml/min.

The HPLC system was coupled to a Perkin-Elmer Sciex API III plus triple quadrupole mass spectrometer equipped with a source for atmospheric pressure ionisation and an articulated ionspray interface (Sciex, Thornhill, Canada). The HPLC eluate was splitted in a 40:1 ratio. The mass spectrometry measurements were carried out under the following experimental conditions: ionspray voltage (ISV), 5.5 kV; orifice voltage (OR), 60 V; scan range for full scan spectra, m/z 200–600, step 0.1 u, dwell time 1 ms; scan rate, 0.24 scan/s; no interscan delay;

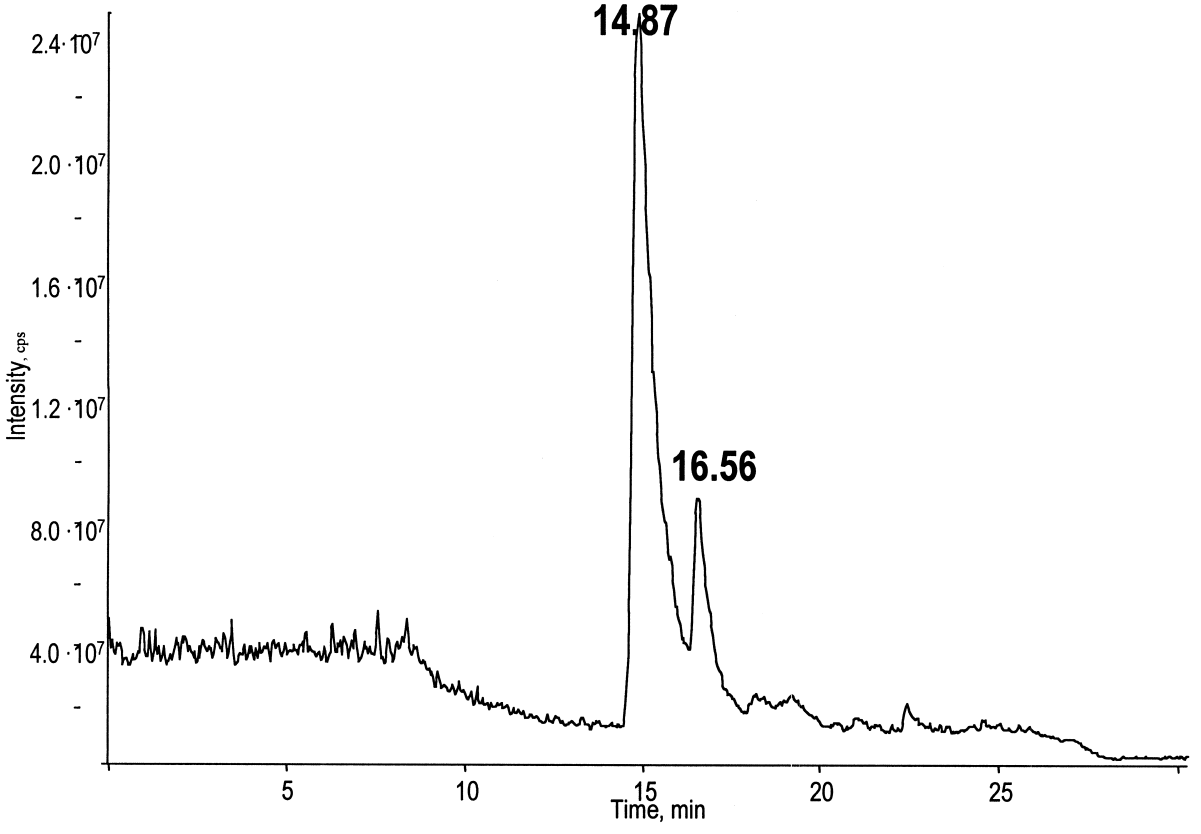

Fig. 2. Total ion current chromatogram relative to the HPLC–MS analysis of the reaction mixture obtained from the iodination of methylene blue.

resolution >1 u. For selected ion monitoring (SIM) acquisitions the molecular cations of the possible species were monitored, at m/z 228, 242, 256, 270, and 284 for the starting 3,7-bis-amino-phenazathioniums **1**–**6**, 354, 368, 382, 396, and 410 for the mono-iodinated species and 480, 494, 508, 522, and 536 for the bis-iodinated species, that is 15 ions with 100 ms dwell time, for a scan rate of 0.62 scan/s.

2.3. Iodination of methylene blue using KI carrier

A iodate/iodide potassium solution (KIO_3/KI, 1 ml) at a final concentration KIO_3 0.018 *M* and KI 0.525×10^{-3} *M* was prepared by adding the KI solution to the KIO_3 solution. A 0.5 ml aliquot of a diluted hydrochloric acid solution was prepared by injecting 1.8 ml of 37% concentrated hydrochloric acid (10.15 *M*) into a unopened septum sealed 100 ml vial of water for injection. A 0.5 ml aliquot of an aqueous solution of 1% **1**(FU IX) was used as supplied by S.A.L.F. The final concentrations in the reaction mixture (2 ml total volume) were: **1**, 0.008 *M*; KI, 0.262×10^{-3} *M*; KIO_3, 0.009 *M*; HCl, 0.042 *M*. The solution was transferred into the sealed vial and placed in the heating block at 100°C for 30 min. At the end of the heating process the vial was left in cold water for 5 min. The reaction mixture was then purified by ion-exchange chromatography by eluting it on a disposable Isolute (IST, Isolute Sorbent Technology, Mid-Glamorgan, UK) column, previously solvated with 5 ml of water. The column was eluted with 4 ml of water and the eluted mixture was directly analysed by HPLC–MS.

2.4. Iodination of methylene blue without carrier

The iodination reaction without carrier was carried out with the same procedure described above except that the KI final concentration was 8.81×10^{-6} *M*.

2.5. Iodination of azure B with and without KI carrier

Azure B (0.1% solution) iodination was carried out following the same procedure described above for methylene blue. Final reaction mixture volume was 2 ml and final concentration of azure B was 0.8×10^{-3} *M*.

3. Results and discussion

The Fig. 2 shows the total ion current (TIC) chromatogram obtained. We can observe two main peaks. The corresponding ISI mass spectra are reported in Fig. 3. As we can see, the major peak, at 14.87 min (Fig. 2a), is attributable to unreacted **1**as expected (the reaction is carried out in a large excess of 1, as in its normal application the expensive reagent is the radioactive iodide), but the minor peak, at 16.56 min (Fig. 2b), shows a molecular mass of 396, 14 Da lower than expected. This compound can be identified as a demethylated product. This finding could be attributed both to a demethylation process occurring during the iodination reaction or to the presence, in the starting material **1** of a demethylated impurity, which is azure B, **2**, whose iodination rate is much faster with respect to **1**. Blower et al. [12] also found the prevalence of iodinated **2** in the reaction mixture, and they attributed such a component to a demethylation reaction occuring during the iodination process. Indeed they did not check the starting methylene blue samples. In order to decide which was the right interpretation, we can check if the expected iodinated derivative of **1** is present in the reaction mixture and if some azure B is present in the starting samples of **1**. Fig. 4 shows the ISI spectrum of the peak at 19.26 min (a) and the extracted ion chromatrogram (XIC) relative to the ion at m/z 410 (b), indicating that the iodinated derivative of **1** is present in the reaction mixture. On the other hand, Fig. 5 shows the ISI mass spectrum of the starting sample of **1** used for the iodination reaction. We can observe the presence of an ion at m/z 270, attributable to the impurity azure B. Moreover, SIM acquisitions show, after integration of the XIC chromatograms, that **2** was present at about 7% (with respect to the **1**+**2** mixture) in the starting samples of **1**, whereas after the iodination reaction **2** only accounts for 4% of the unreacted dyes. All these observations clearly lead to the conclusion that the reaction proceeds mainly with the iodination of **2**, and **1** is iodinated only in a minimal amount. This behavior can be explained on the basis

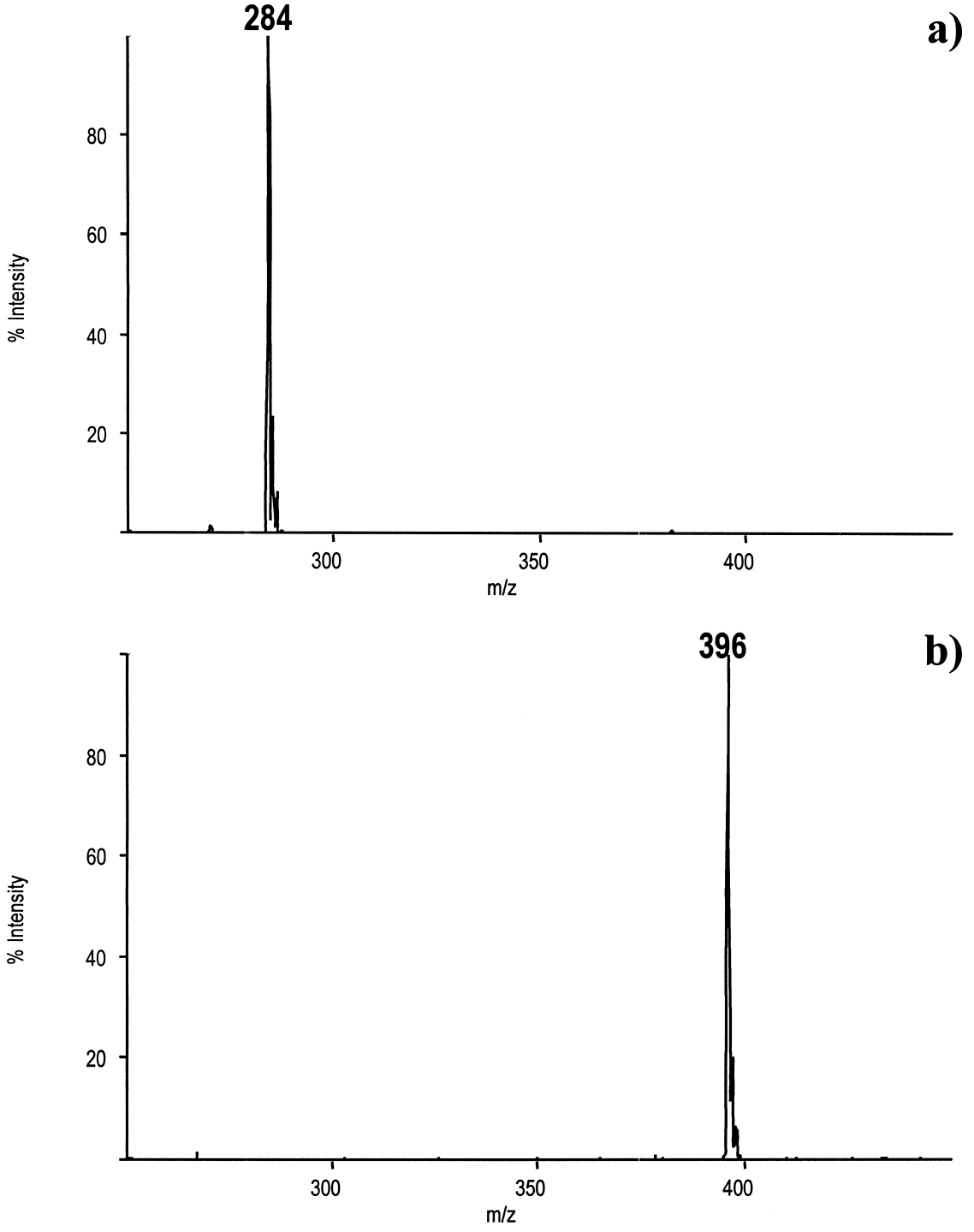

Fig. 3. ISI mass spectra corresponding to the peaks at 14.87 min (a) and 16.56 min (b) in the chromatogram shown in Fig. 1.

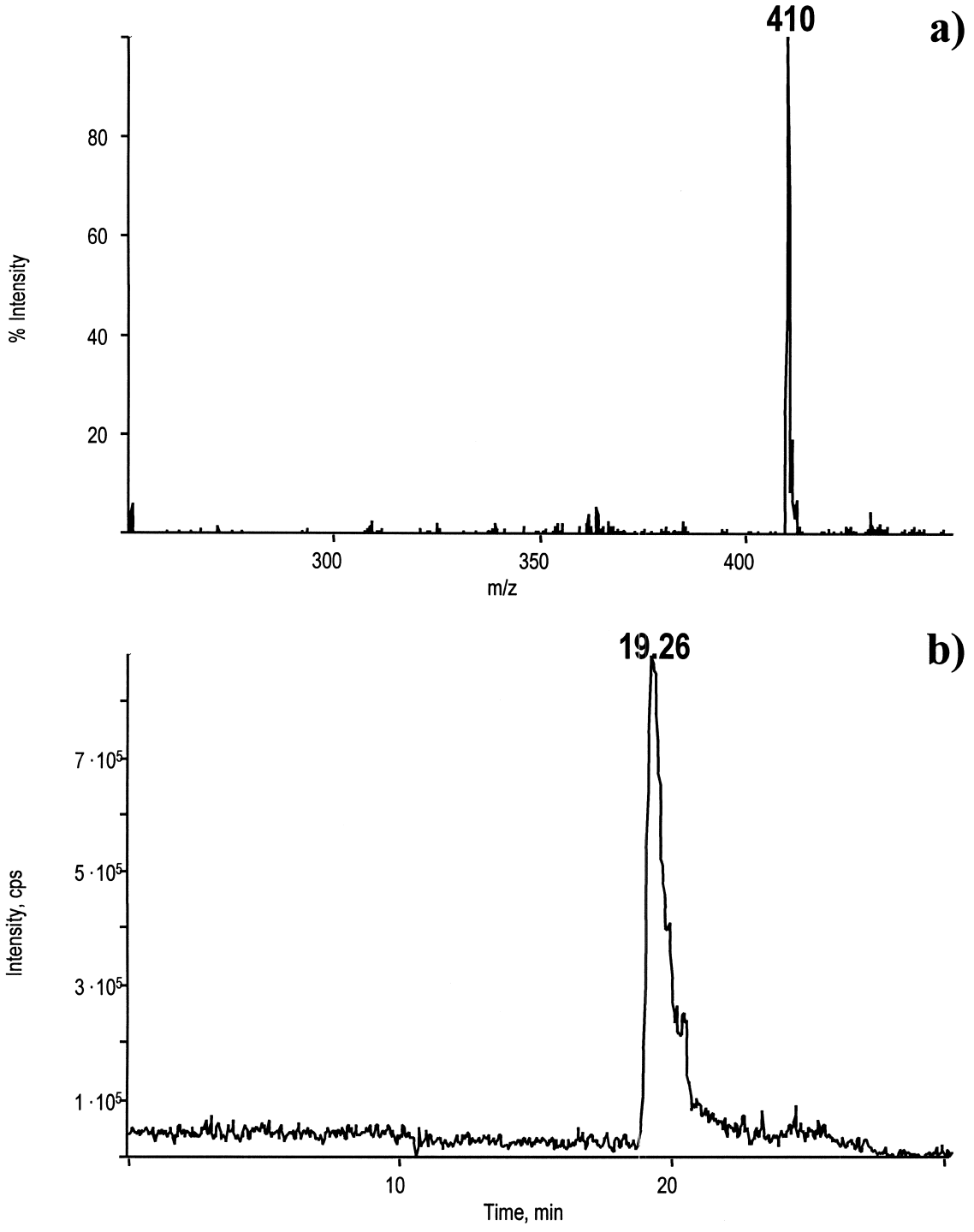

Fig. 4. ISI mass spectrum corresponding to the compound eluted at 19.26 min (a) and XIC chromatogram relative to the ion at m/z 410.

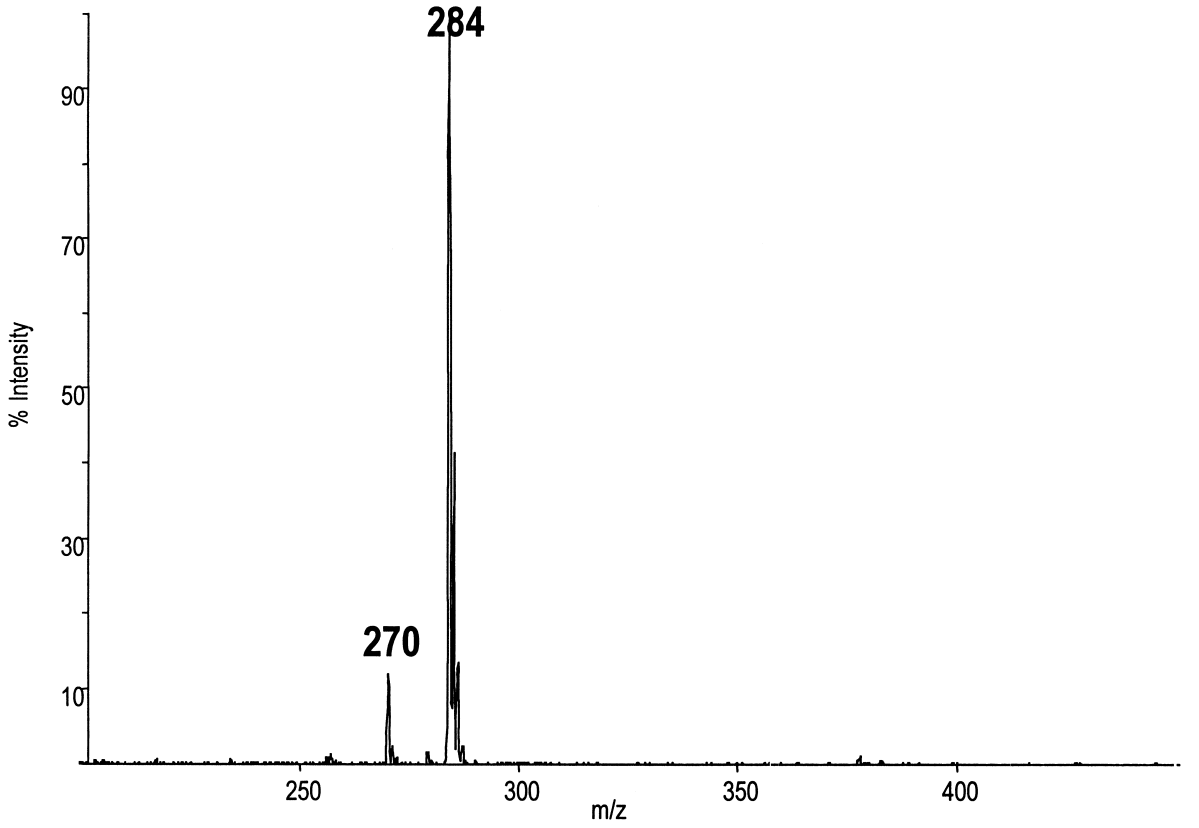

Fig. 5. ISI mass spectrum of the methylene blue used as a substrate for the iodination reaction.

of the different steric hindrance between the iodine atom and the dimethylamino (in **1**) or the methylamino (in **2**) group in the formation of the Wheland intermediate of the aromatic electrophilic substitution, as well as in the Wheland intermediate itself and in the final product. The same steric effects can also explain the absence of any doubly iodinated products in the reaction mixture.

The presence of **2** as an impurity in samples of **1** (we tested also other batches coming from the same supplier as well as samples supplied by different companies) induced us to investigate also the iodination of **2**, in order to get a better insight of this iodination process, and also in view of a possible use of azure B in place of methylene blue, at lower dosages, for radiodiagnostic purposes. We tested different samples of **2**, coming from different suppliers, and we found out that they contain **1** as an impurity, but also the compounds with all the possible methylation grades shown in Fig. 1 (**3**–**6**). Fig. 6 shows the TIC and the XIC chromatograms relative to the molecular cations of the products **1**–**6** for a commercial azure B sample. The iodination of this sample was carried out in the usual conditions used for **1** and the reaction mixture was analysed by HPLC–MS in the SIM mode. Fifteen ions were monitored and in particular m/z 284, 270, 256, 242, 228 (for compounds **1**–**6**), 410, 396, 382, 368, 354 (for their mono-iodinated products, respectively), and 536, 522, 508, 494, 480 (for the possible bis-iodinated products, if any). The compounds **3** and **4** are poorly separated under the conditions adopted, so they are considered together. The iodination mixture of **1** was examined in the same conditions. For both the substrates the reaction was performed in the presence or absence of potassium iodide carrier. The TIC chromatograms relative to the four samples are reported in Fig. 7. As their precursors, also the

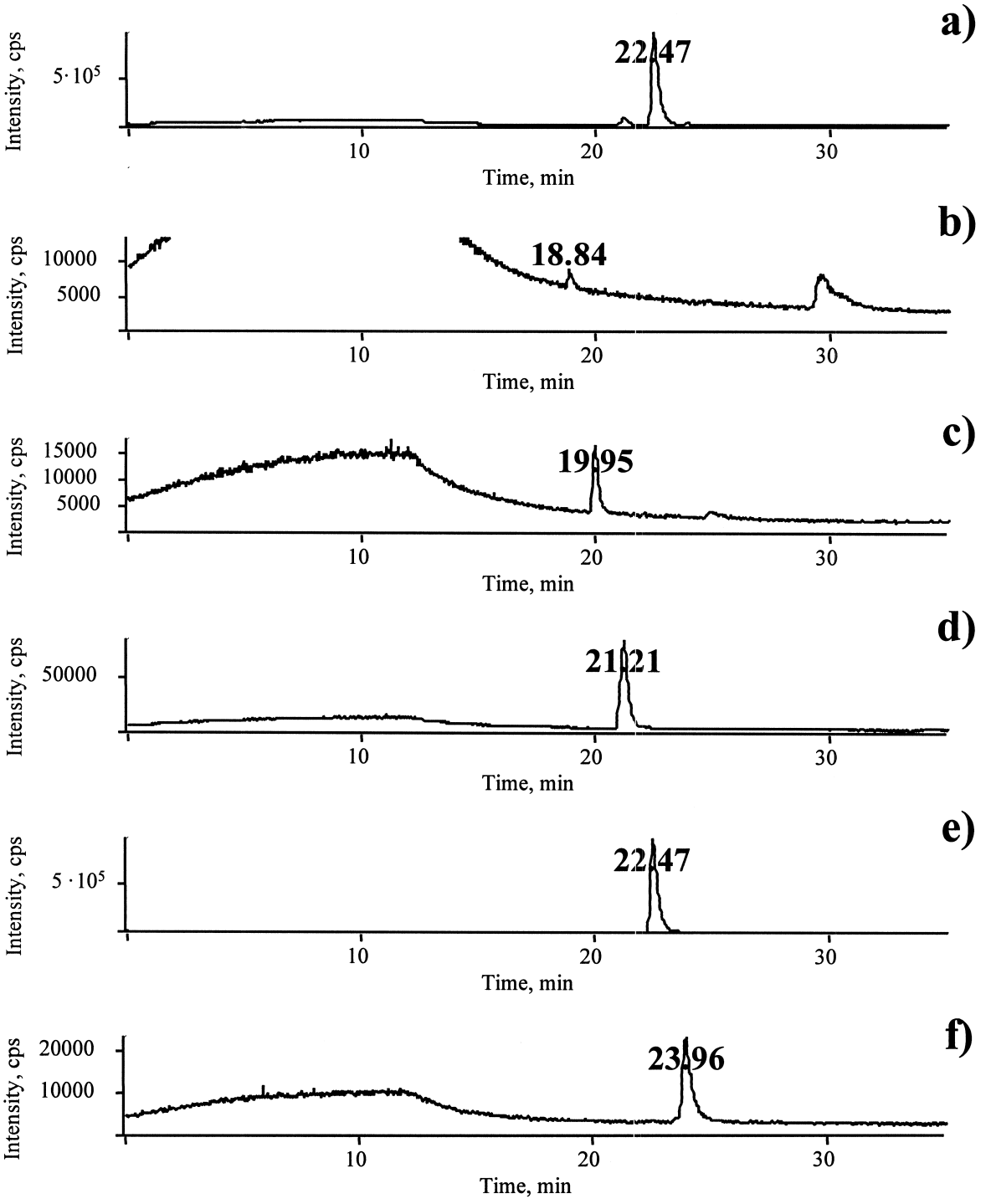

Fig. 6. Total ion current (a) and XIC chromatograms relative to the ions at m/z 228 (b, compound **6**), 242 (c, compound **5**), 256 (d, compounds **3** and **4**), 270 (e, compound **2**), and 284 (f, compound **1**) obtained in the HPLC–MS analysis of a commercial sample of azure B.

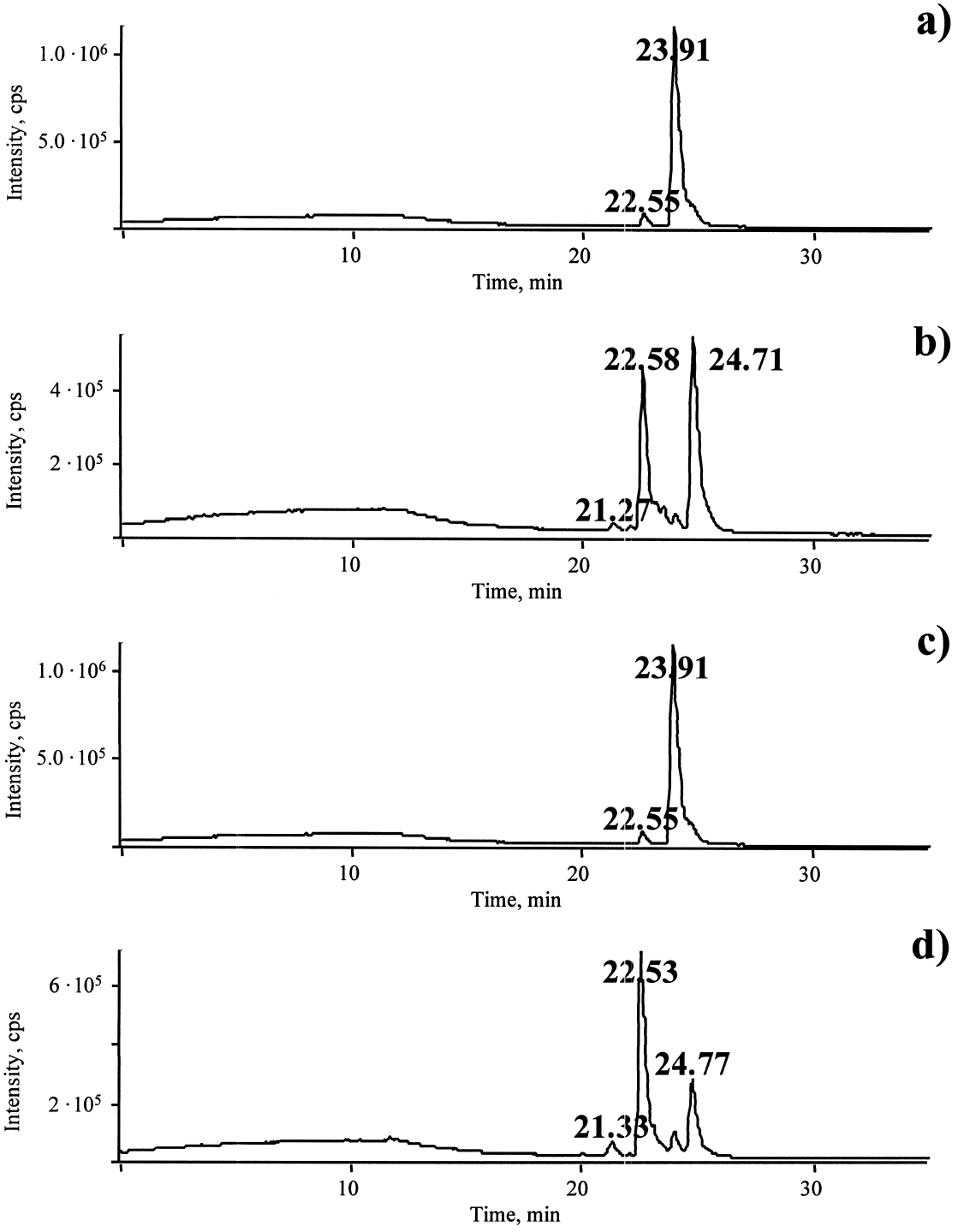

Fig. 7. Total ion current chromatograms relative to the HPLC–MS analysis of the reaction mixtures obtained from the iodination of methylene blue (a) and azure B (b) in the presence of potassium iodide carrier and (c and d) in the absence of potassium iodide carrier.

iodination products of **3** and **4**, **3**-I and **4**-I, are poorly separated, even if slightly better than the precursors. Also these iodination products are considered together.

As the cations have a very similar structure and the charge is already present on the molecule, we can think that the ISI–MS responses of the 15 different species should be very close. Under this approximation, the integration of the XIC chromatograms can provide a good estimation of the relative amounts present in the final reaction mixture. We used manual integration using the integration routine available in Multiview 1.3, the data reduction program of the Sciex API data system. The results are summarised in Table 1.

As we can observe, double iodination is very unlikely under the reaction conditions adopted. No traces of bis-iodinated compounds of **1**, **2**, and **6** could be detected and only traces of **3**/**4**-I_2 (0.02% for methylene blue, 0.15% for azure B) and **5**-I_2 (0.05% only for azure B) were found in the reaction mixtures. The effect of steric hindrance is confirmed, as we can deduce by comparing the reactivity of **3**/**4** and **2**. As far as the effect of potassium iodide carrier is concerned, we can say that its presence improves the extent of the iodination reaction.

On the basis of the above results, we can conclude that the rate of the iodination reaction is strongly reduced by the presence of methyl groups on the amino substituents of the aromatic ring. Double iodination occurs only for the dimethyl derivative, very likely for the symmetric one, **4**, and for the monomethyl derivative **5**, if present in a sufficient concentration. This strong influence of steric effects suggest that the iodination position is the 2 (or 8 for unsymmetrical derivatives) as the positions 4 and 6 (peri positions) are more hindered as usual for condensed aromatic rings.

4. Conclusions

In conclusion, in the iodination of methylene blue the main iodinated product is the azure B, which is 10–15 times more abundant in the iodination mixture of samples of methylene blue, containing azure B as an impurity at levels of 7–8%. This behaviour can be explained on the basis of steric hindrance between the entering iodine atom and the methyl groups present on the amino substituents, both in the formation of the Wheland intermediate (kinetic control) and in the final products (thermodynamic control). For the same reason, dual iodination occurs only for the symmetric dimethyl derivative **4** and, if present in sufficient concentration, for the monomethyl derivative **5**.

The results described in the present paper suggest a possible use of azure B in place of methylene blue, which could be given at much lower (10–100 times lower) dosages for diagnostic purposes. Studies are in progress to check this possibility. The present investigation also indicates that HPLC–ISI–MS can be very useful also for in vivo investigations in order to help in the elucidation of the distribution of the drug in the organism and of its pharmacokinetic and pharmacodynamic profiles.

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Table 1
Composition of the starting substrates and of the reaction mixtures in the iodination reaction of methylene blue and azure B

Sample	**1** (%)	**2** (%)	**3**/**4** (%)	**5** (%)	**6** (%)	**1**-I (%)	**2**-I (%)	**3**/**4**-I (%)	**5**-I (%)	**6**-I (%)	**3**/**4**-I_2 (%)	**5**-I_2 (%)
Starting **1**	92.17	7.19	0.64	–	–	–	–	–	–	–	–	–
Starting **2**	2.45	89.27	7.15	0.93	0.20	–	–	–	–	–	–	–
Iod. Mixt. **1**/KI	89.45	3.79	0.11	–	–	0.36	5.69	0.53	–	–	0.02	–
Iod. Mixt. **2**/KI	3.37	35.78	1.82	0.15	–	0.07	50.75	6.79	0.88	0.19	0.15	0.05
Iod. Mixt. **1**	89.60	3.99	0.16	–	–	0.35	5.34	0.56	–	–	–	–
Iod. Mixt. **2**	7.17	59.61	4.13	0.40	0.12	0.05	24.23	3.74	0.59	0.03	0.03	–

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